Arsenic, Lead and Manganese as Risk Factors for Child and Maternal Neurotoxicity

ARSENIC, LEAD AND MANGANESE AS RISK FACTORS FOR CHILD AND MATERNAL NEUROTOXICITY

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ABSTRACT

Metals such as arsenic, lead and manganese are naturally occurring elements readily found in the earth's crust. Numerous studies have shown that these metals can be neurotoxic though the exact mechanism remains unknown.

In our neurobehavioral study, we found a significant association between prenatal lead concentrations and the scores of Adaptive Skills domain of the BASC-2, β(SE) = -5.99 (2.68), p-value 0.025. Positive cognitive home environment was consistently associated with better neurobehavioral outcomes. In our investigation, we found that arsenic was significantly associated with increased postpartum depression in mothers in the Tar Creek cohort. β (SE) = 0.97 (0.44). The association was statistically significant (p =0.027). We also found a significant interaction between lead and arsenic (β = 1.49 (0.62), p=0.017). Results of an epigenetic study suggest that mothers of children with neural tube defects may have different maternal plasma histone levels than unaffected children. We found a significant association, β (SE) = 0.041 (0.014), p =0.006, between H3K27me3 levels and NTD case status. Among mothers with low folate, H3 was negatively associated, β (SE) = -10.5 (4.05), p =0.016 with maternal arsenic exposure.

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INTRODUCTION

Heavy Metals

Metals such as arsenic, lead and manganese are globally ubiquitous. Exposure primarily occurs through contamination of drinking water, direct exposure to soil, and inhalation of dust. Anthropogenic processes such as mining and other industrial processes often serve as a means of contaminating local soil and water supplies. While occupational studies have focused on high-level exposure, numerous studies have shown that these metals are neurotoxic even at low levels. Increasingly, investigations are focusing on neuropsychological outcomes. It is important to note that while metals such as lead and arsenic have no nutritional benefits, manganese is an essential nutrient.

Lead

Lead exists naturally in the Earth's crust at low levels. However, human activity has resulted in high levels of environmental lead. Sources of lead include mining, lead-based paint and solder, waste incineration and coal combustion Lead exists in 2+ and 4+ oxidation states with the majority as 2+. The presence of lead minerals correlates with zinc, copper and iron sulfides (Reeder et al, 2006).

Environmental estimation of lead exposure is primarily obtained through water and soil sampling. Use of water samples offers direct comparison to a long body of research on the health effects of lead exposure. EPA guidelines for lead are based on water sampling, which lends itself to the immediate application to regulatory controls.

Biomarkers of lead exposure include bone, blood and urine. Lead metabolism and excretion offers advantages to utilizing various methods of estimating exposure. Most of the lead absorbed by the body ends up in mineralized tissue (e.g. bone and teeth). The half-life ($t_{1/2}$) of

lead in bone is approximately 27 years (Reeder, 2006) and therefore serves as an ideal biomarker of chronic exposure. However, the complexity of obtaining bone and teeth makes their availability as biomarkers limited. However, the $t_{1/2}$ of lead in the blood is approximately 30 days. This duration certainly allows for the observation of acute exposure but may also represent chronic exposures, assuming steady state. Lead is also excreted through urine and feces (ATSDR, 2007).

There are multiple mechanisms of lead neurotoxicity. Inorganic lead often forms complexes with a variety of ligands, such as proteins in the cell nucleus, cytosol and red blood cells. One major mechanism is through the disruption of calcium function. Calcium, an important cofactor, is involved in cellular processes including cell-signaling pathways e.g. protein kinase C (PKC) pathway. This pathway is involved in the synthesis of neurotransmitters, operation of ion channels and dendritic branching. Lead also changes the activity of an important calcium receptor called calmodulin. In addition to calcium, lead also interferes with another important cofactor, zinc. Lead can sometimes substitute zinc in enzyme and zinc-finger proteins. The resulting substitution can lead to abnormal expression of these proteins and well as the gene transcription regulated by these proteins. (ATSDR 2007, Bouton et al. 2000, Bressler et al. 1999). Finally, lead can alter the glutamatergic, dopaminergic and cholinergic systems of the brain (Cory-Slechta, 1995).

The effects of lead toxicity have been documented for centuries. However, scientists in the past few decades have found that even low-level lead exposure can have detrimental health effects from early life through late life. In Chapter 2, I will focus my investigation on early life (including prenatal) exposures to lead in children and neurodevelopment. In Chapter 3, I will focus on exposures in mothers and mental health.

Arsenic

The average concentration of arsenic in the Earth's crust is approximately 2 ppm (Wedepohl, 1995). Common sources of environmental arsenic include pesticides, herbicides, waste from mining and industrial smelting activities and animal waste from additives in poultry feed (Reeder, 2006). These sources of arsenic often find their way into groundwater and soil.

Inorganic arsenic (iAs) exists in five oxidative states: 3-, 1-, 0, 3+ and 5+. The most common environmental states are arsenite (3+) and arsenate (5+). Arsenic metabolism occurs primarily in the liver. The metabolic pathway of arsenic is thought to occur through oxidative methylation and glutathione conjugation (Watanabe et al, 2013). During oxidative methylation, arsenic is monomethylated (MMA) or dimethylated (DMA) by arsenite methyltransferases using S-adenosylmethionine (SAM) as a methyl donor (Abernathy, 1999). Inorganic arsenic is more toxic than organic arsenic - in order of decreasing toxicity: arsenite > arsenate >MMA > DMA. Methylation is, in a sense, a mechanism of arsenic detoxification. Arsenic speciation provides important insight into the sources and mechanisms of toxicity.

Estimates of arsenic exposure are typically obtained through environmental water sampling and/or through biomarkers. Typical matrices for biomarkers of exposure include blood, hair, urine and nail. Again, metabolism and excretion of arsenics offers advantages to utilizing certain biomarkers. Elimination of arsenic from the body is primarily through urine. Arsenic tends to accumulate in protein rich tissue such as hair and nails over time (Mandal et al, 2003, Rabal et al 2005). This allows for the observation of continuous exposure making them ideal biomarkers for chronic arsenic exposure. However, environmental contamination of hair and nails requires stringent cleansing before analytical analysis. Use of urine and blood as

biomarkers avoids the problem of pervasive environmental contamination, but only allows for analysis of acute arsenic exposure.

The two primary proposed mechanisms of action for arsenic toxicity are the formation of reactive oxygen species (ROS) and oxidative stress. ROS are ions or molecules produced by the partial reduction of oxygen. ROS, which alter protein structure and function, are important to many cellular processes such as gene expression and signal transduction. The mitochondria produce endogenous ROS. An imbalance of ROS can result in macromolecular damage and contribute to disease pathology (Ray et al, 2012). Arsenic-induced ROS include hydrogen peroxides (H_2O_2), superoxides ($O_2^{\bullet -}$), hydroxyl radicals ($^{\bullet}OH$) and singlet oxygen ($^1O_2$) (Flora, 2011).

Oxidative stress refers to the damage that arises from redox imbalances. This imbalance is the result of the generation of excessive free radicals and/or the dysfunction of the antioxidant system (Kandola et al, 2015 and Kim et al, 2015). While there are many types of free radicals, ROS are the most relevant. Though oxidative stress primarily occurs through ROS-induced pathways, it is not necessarily the case.

Scientists believe that arsenic induces oxidative stress by several mechanisms. First, the generation of an arsinine intermediate that can produce free radicals. Second, methylated arsenic can cause the release of iron from ferritin leading to the production of ROS. Finally, oxidative methylation of arsenic (reduction of As(V) to As(III)) can also generate ROS. ROS also causes damage directly to DNA (Flora, 2011). Flora also asserts that the generation of ROS remains the key event in arsenic-induced disruption in cell signaling pathways. An exploding area of interest is the investigation of the role of epigenetic mechanisms in arsenic toxicity. I will explore these mechanisms in Chapter 4 of my dissertation.

Manganese

Manganese is the 12th most abundant element and makes up approximately 0.1 % of the earth's crust. Manganese has several oxidation states and can exist in both organic and inorganic forms. While manganese appears as Mn(III) in several enzymes (Leach et al, 1978 and Utter 1976), the primary forms of environmental manganese are Mn(II) or Mn(IV). This suggests that inorganic manganese undergoes changes in oxidation states within the body (Gibbons et al, 1976). Elemental Mn does not naturally exist but rather is found as a complex. Primary environmental sources of manganese are food, soil, air and water (ATSDR, 2012).

Unlike arsenic and lead, manganese is an essential nutrient necessary for human health. Manganese is involved in various enzymatic reactions. It necessary for many processes such as immune function, bone growth and blood coagulation, cellular energy and protection against oxidative stress (Horning et al, 2015). Manganese deficiencies in humans are rare. However, excess exposure to Mn is neurotoxic.

While manganese is essential for protection against oxidative stress, ironically, Mn neurotoxicity may also occur through redox mechanisms. It can alter transport mechanisms, receptor function and enzyme activity (Horning et al, 2015). The primary target of manganese toxicity is the central nervous system where it accumulates in the basal ganglia of the brain. While the exact mechanism of Mn neurotoxicity is unknown, studies suggest manganese neurotoxicity may lead to damage by increasing levels of extracellular glutamate. It may also affect dopamine metabolism leading to behavioral changes (Fitsanakis et al, 2006). Though manganese is rapidly excreted in bile through feces, the primary biomarkers of manganese exposure are blood and urine. Both matrices are considered to represent recent exposures (ATSDR, 2012).

Neurological Health

Neuropsychology of Children

Neurobehavioral development disorders affect 10-15% of births in the United States (Bloom et al, 2010). Genetic factors account for only 30-40% of cases. Thus, environmental factors and their interactions with genetic predisposition are important in the causation of neurobehavioral disorders (National Research Council, 2000).

Studying neurodevelopment as an endpoint is particularly complex. Social environment is generally regarded as cofounders when investigating the association between environmental toxicants and neurodevelopment. However, early toxic exposures can alter the way a child interacts with their social environment, which in turn affects their neurodevelopment (Bellinger, 2009). Thus a complex dynamic exists that must be considered when investigating the association between a child's environment and neurodevelopment. Our understanding of the impact of the environment on neurodevelopment is crucial in diagnosing and treating the needs of the growing child.

Children are an extremely susceptible population. The developing human brain is particularly vulnerable to environmental insults. Critical windows of exposure include *in utero* and infancy. Thus, these exposures often set the stage for poorer health later in life.

The placenta does not protect against many chemical exposures (Needham et al, 2011). A study headed by the Environmental Working Group (EWG) found an average of 200 exogenous chemicals in the umbilical cord of 10 babies (EWG, 2005). Additionally, chemicals can be transferred from mother to child through breast milk. During infancy, the blood-brain barrier provides little protection from these neurotoxic chemicals (Zheng et al, 2003).

Environmental toxicants, even at low dose, can cause permanent brain damage. For example, methylmercury has detrimental effects on the developing brain at doses that would not be harmful to the adult brain (Oken et al, 2008). Recent studies found that increased levels of serum dichlorodiphenyltrichloroethane (DDT) and dichlorodiphenyltrichloroethylene (DDE) are associated with decreased neurodevelopmental function (Torres-Sanchez et al, 2013; Boucher et al, 2013).

There are multiple instruments available to assess the neurological health of children. A common method of assessing neurocognitive function is intelligence tests. A widely used test of intelligence is the Wechsler Preschool and Primary Scale of Intelligence – Third Edition (WPPSI-III). In addition to assessing neurocognitive function, there are also instruments to study neurobehavioral outcomes. The Behavior Assessment Scale for Children (BASC-2) is a general measure of behavior (Kamphaus and Reynolds, 1999). The BASC-2 measures emotional and social function of children from preschool to high school. The Behavior Rating Inventory of Executive Function-Preschool (BRIEF) evaluates executive function in children. While various definitions of executive function exist, general consensus it that executive function is the control and self-regulatory functions that allow the organization and direction of behavior, emotional response and cognitive activity (Gioia et al, 2004). A detailed description of the BASC-2 and BRIEF scales can be found in Chapter 2.

Postpartum Depression

Depression is the fourth leading cause of disability in the world (Stranieri et al, 2013). Based on NHANES data, 1 out of 20 people in the United States, over the age of 12 reported having depression between the years 2005-2006 (Pratt et al, 2008). 6.7% of females reported having depression compared to 4% of men. This difference was statistically significant. In

Chapter 3 of my dissertation, I will focus on postpartum depression in mothers from the Tar Creek Cohort.

Postpartum depression (PPD) is a non-psychotic depressive episode that affects 10 to 15% of new mothers worldwide (Elisei et al, 2013 and Patel et al, 2012). The Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) defines PPD as a sub-category of major depressive disorder. PPD begins within 4 weeks post-delivery and can extend into the first postnatal year.

While the exact cause of PPD is unknown, most studies attribute the cause to major hormonal changes during pregnancy. Levels of estrogen, progesterone and cortisol begin to change rapidly within the first 48 hours of delivery. Risk factors for PDD include stress, lack of social support and family history of mood disorders. However, history of depression (pregnancy related or not) is the single biggest predictor for postpartum depression (Patel et al, 2012).

The Edinburgh Postnatal Depression Scale (EPDS) is one of the most widely used screening tools for PDD. The 10-item questionnaire is efficient and easy to administer. An EPDS score of ≥ 10 (maximum score of 30) is often considered the initial cut-off to recommend new mothers for follow-up mental health screening (Wisner et al, 2002). I will further detail the topic of postnatal depression and the EPDS in Chapter 3 of my dissertation.

Neural Tube Defects and Epigenetics

Neural tube defects (NTD) affect around 1 in 1000 pregnancies and are the number one cause of perinatal mortality and morbidity. The neural tube, the precursor of the brain and spinal cord, begins to form during the 4^{th} week of gestation (Denny et al, 2013). Specialized cells form a neural plate. The plate then forms a tube in two stages: primary neurulation and secondary neurulation. Neural tube defect form when there is some failure in the neurulation process. There

are two classifications of NTDs- open and closed. Types of NTDs include anencephaly, spina bifida and myelomeningocele. The severity and prognosis of the different types of NTD vary.

Genetic, epigenetic and environmental factors play an important role in development of NTDs. Epigenetic mechanisms are critical in embryonic development. The epigenetic mechanisms of DNA methylation, micro RNA and post-translational histone modifications (PTHM) are important in gene regulation. The modifications due to these mechanisms, unlike genetic code, may be reversible. Therefore, a better understanding of these mechanisms may provide hope for therapeutic targets. Much of what we know come from animal studies. Increasingly, epidemiological studies have sought to understand the role of epigenetic mechanism in NTD etiology (Barber et al, 2000). In Chapter 4, I will discuss PTHMs in more detail.

Research Objective

My dissertation seeks to contribute to current literature that investigates the association between metals exposure and neurological health. Much of the information known regarding the effects of metals on neurological health rely on animal studies. Studies in human populations are often conducted in populations with high metals exposure outside of the US. This presents gaps in the current literature pertaining to chronic low-dose exposure of human populations in the US.

Specific Aim 1:

Investigate the association between neurobehavioral outcomes and biomarkers of arsenic, lead and manganese exposure in children age 5-7 in the Tar Creek Study Cohort.

Specific Aim 2:

Investigate the association between postnatal depression and biomarkers of arsenic, lead and manganese exposure in mothers from the Tar Creek Study Cohort.

Specific Aim 3:

Investigate the association between extracellular histones level and biomarkers of arsenic exposure in a pilot case-control study of Neural Tube Defects in Bangladesh.

Overview of Dissertation

My dissertation begins with a review of literature regarding the health effects of lead, arsenic and manganese. I will then provide a brief introduction to the neurological outcomes of interest- neurobehavior in children, depression and histones. My first study will focus on the association between prenatal biomarkers of arsenic, lead and manganese exposure and neurobehavior in children at 5-7 in the Tar creek cohort. My second study focuses on the association between these same metals and postpartum depression in mothers at birth. Finally, my third study on will investigate arsenic toxicity and histone modifications in the context of a case control pilot study investigating neural tube defects. Finally, I will summarize my finding and discuss suggestions for future study.

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CHAPTER II

Metals Exposure and Neurobehavioral Assessment of School-age Children in the Tar Creek Superfund Cohort Study

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ABSTRACT

Background: Arsenic, lead and manganese are known to impact neurological health. Prenatal exposure to these metals can have lasting impact on neurodevelopment – both neurocognitive and neurobehavioral function.

Objective: Our objective was to investigate the association between prenatal arsenic, lead and manganese exposure and neurobehavioral outcomes in school-age children.

Design: We conducted a prospective analysis of the Tar Creek Superfund Cohort Study. The concentrations of metals in cord blood provided estimates of children's prenatal metal levels. At follow-up, around 5 -7 years of age, 122 children were given neurodevelopmental assessments. We will focus on neurobehavioral function as assessed by two parent-completed questionnaires, the Behavior Rating Inventory of Executive Function-Preschool and the Behavior Assessment Scale for Children – Second Edition.

Results: We found a significant association between prenatal lead concentrations and the scores of Adaptive Skills domain of the BASC-2 (β = -5.99, p-value 0.025). Cognitive HOME (CHS) scores were generally found to have significant negative associations, except with Adaptive Skills in which the associations were positive. Maternal IQ and child age was found to be significantly associated with the BSI and Adaptive Skills for all metals. In our lead models, we found that boys had significantly higher ISCI scores (β = 5.31, p-value 0.005) and externalizing problems (β = 8.40, p-value 0.021) than girls. The same association was found in our arsenic and lead models.

Conclusion: This paper is a follow-up to studies on the neurodevelopment of children in the Tar Creek Cohort. Increased blood lead levels at birth were significantly associated with worse

adaptive skills assessed by the BASC-2 using our multivariable model. We found no significant associations between other blood metal concentrations and BRIEF or BASC-2 scores.

INTRODUCTION

Arsenic, lead and manganese are metals found globally in the earth's crust. While manganese is a micronutrient, lead and arsenic have no known benefit while exhibiting various toxicological effects. Numerous studies have investigated the neurological effects of these metals.

Neurodevelopment: Cognitive and Behavioral Function

It is critical to understand how dosage and timing of exposure affect their outcomes. Childhood is a critical window to investigate due to the vulnerability during neurodevelopment. Lead is perhaps the most well characterized metal with known neurotoxicologial effects in children. However, more studies are exploring the effects of other metals such as manganese and arsenic on neurodevelopment.

A cross-sectional study by Calderón et al (2001) investigated the effects of long-term exposure to lead and arsenic on intelligence in Mexican children. The study found a significant decrease in verbal IQ with increasing urinary arsenic (UAs) measures. Higher arsenic exposure was also associated with poorer performance on tests of memory and language. Wasserman et al (2004) investigated the impact of arsenic on the intelligence of 10-year-old children in Bangladesh. The study found that children in the third and fourth quartiles had significantly lower Full-Scale and Performance IQ (raw scores) compared to children in the first quartile with p-values of <0.05 and <0.01 respectively. Another study by Wasserman et al (2007) found similar trends in 6 -year old Bangladeshi with children. A study by Rosado et al (2007) of Mexican schoolchildren investigated cognitive performance. The study found that children with

UAs of >50 µg/L performed significantly lower ($p<0.05$) on math tests, visual tests and WISC-RM Digit Span subscale tests compared to children with UAs concentrations <50 µg/L. Using the WISC, a study by von Ehrestein et al (2007) found that increasing UAs concentrations were associated with decreased performance on vocabulary, object assembly test and picture completion tests among West Bengal children ages 5-15. A 2011 study by Hamadan et al showed that increasing UAs was significantly associated with decreased verbal and full-scale IQ in Bangladeshi girls. However, the same association was not present in boys. Another study by Roy et al, 2009 also investigated the association between metals and behavior in Indian children. Investigators found that blood lead was associated with higher anxiety ($p=0.01$), social problems ($p=0.02$), global executive function ($p<0.001$) and attention deficit ($p=0.05$).

This paper builds upon the results of a cross-sectional pilot study by Wright, et al in 2006, which explored the association between metals exposure and cognitive function and behavior in thirty-two children age 11-13. Hair arsenic, manganese and cadmium were the exposure metrics. The outcome instruments used in this study included the Wechsler Abbreviated Intelligence Scale (WASI), Wide Range Assessment of Memory and Learning (WRAML), Wide Range Assessment of Visual Motor Abilities (WRAVMA), Behavior Rating Inventory of Executive Function-Preschool (BRIEF), Behavior Assessment Scale for Children (BASC), and the Conners' ADHD DSM-IV Scales (CADS-IV). Arsenic exposure was found to be significantly associated with lower scores on the full-scale IQ ($\beta = -0.44$, $p= 0.01$) and verbal IQ ($\beta= -0.51$, $p= 0.003$) tests. Additionally, significant interactions were found between As and Mn on some memory tests.

A neurocognitive study by Claus-Henn (2010) from the Tar Creek Cohort demonstrated the importance of considering metals mixtures. The study showed a significant interaction

between manganese and lead. The study found a negative association between Bayley mental and psychomotor development scores in children and lead-manganese exposure (β = -1.27 and -0.92 respectively).

The current study provides a follow-up to the Claus-Henn study, which assessed neurodevelopment in children 1-2 years of age. In our current study, we primarily focus on the neurobehavioral outcomes of children age 5 to 7. We assess general measures of behavior using the BASC-2 and BRIEF in order to characterize the neuropsychological effects of metal exposures further in this population.

METHODS

Study Population

The study population has been previously described (Ettinger, 2009). Briefly, the Tar Creek Metals Assessment Targeting Community Health (MATCH) study is located at the Tar Creek Superfund site. It is a former mining site located in northeast Oklahoma. In 1983, the EPA added the site to the National Priority List due to extensive metal contamination of water and soil. Approximately 30,000 people live in the area with some residents being of Native American descent (Ettinger, 2009). According to the U.S. Geological Survey in 2007, 25% of the drinking water samples contained levels of arsenic that were above the EPA concentration limit of 10 µg/L.

The Tar Creek MATCH study is a prospective birth cohort to study biological markers of early life exposure to environmental toxicants. Data collected includes metals exposure, stress indicators and infant growth. Subjects recruited were pregnant women giving birth at Integris Hospital in Miami, Oklahoma who planned to live in the area for the next two years. Subjects also had to illustrate English language proficiency and did not have another child already

enrolled in the study. Information on sociodemographic characteristics, potential sources of exposures and psychosocial stress was collected using interviewer-administered questionnaires. Hospital staff collected anthropometric information such as height and weight at birth. Maternal blood samples and cord blood samples were collected within a 12-hour window of delivery birth. Data for 713 children at birth is available for analysis. Follow-up visits included the ongoing collection of this data (Zota, 2009 and Ettinger, 2009). Follow-up collected some covariate measures at 3-month intervals. Neurodevelopment indicators were measured at ages 1, 2 and 5 to 6 years of age. Mental health indicators were measured every six months from baseline to two years.

Exposure Assessment

Maternal samples collected at delivery include whole blood, hair and urine. Child samples collected at delivery include cord blood and hair samples. Additional samples of hair, nails and urine were collected from mothers at six months intervals up to 24 months, and hair and nails for children. The HSPH Trace Metals Laboratory analyzed arsenic levels using inductively coupled plasma mass spectrometry (ICP-MS). The limit of detection (LOD) for whole blood arsenic using this technique is 0.2 μg/L while the LOD for hair is 0.2 ng/g (Ettinger, 2009). Quality control measures include external calibration with standard reference materials, use of an indium internal standard and analysis of replicates.

Instruments for Child Neurobehavioral Function

Behavior Rating Inventory of Executive Function-Preschool (BRIEF)

The BRIEF measures executive function in children 2 to 18 years of age. Executive function refers to the behavior, emotional and cognitive functions that are involved in problem solving. (Papazoglou et al, 2013). We utilized two forms to cover the age ranges: 1) 2 years to 5

years, 11 months 2) 5 to 18 years of age. The form utilized in the Tar Creek cohort is the 2 years to 5 years, 11 months (Preschool Version). The BRIEF is an 86-item questionnaire administered to parents and teachers (Isaacs and Oates, 2008) and asks questions regarding child behavior and personality. The questionnaire takes ten to fifteen minutes to complete. Sub-domains of the tests are Inhibit, Shift, Emotional Control (EC), Working Memory (WM) and Plan/Organize PO). The Inhibitory Self-Control Index (ISCI) combines the Inhibit and EC raw scores (Gioia and Isquith, 2004). The Flexibility Index (FI) combines the Shift and EC raw scores. The Emergent Metacognition Index (EMI) combines the WM and PO raw scores. The Global Executive Composite combines the raw scores of all five sub-domains. For all scales, a higher score suggests greater difficulties in executive function. BRIEF assessment has high internal consistency with alphas of 0.80 to 0.98. The test-retest reliability coefficient for parents was 0.82 (Gioia, 2000).

Behavior Assessment Scale for Children – Second Edition (BASC-2)

The BASC-2 measures emotional and social function of children from preschool to high school. Composite scores are Internalizing Problems, Externalizing Problems, Behavior Symptoms Index and Adaptive Skills. The instrument used for our study was the preschool version form answered by parents. The parent form has 134 items on a 4-point scale from 0-4 indicating *never* to *almost always*. For the Parent Rating Scale, internal consistency for Internalizing and Externalizing Problems was 0.87 and 0.90 respectively for children age 4 to 5. Test-retest reliability on the parent form for Internalizing and Externalizing Problems was 0.86 and 0.81 respectively (Reynolds, 2004). Higher BASC-2 scores indicate worse emotional and social function with the exception of the adaptive domain in which higher scores indicate better adaptive skills.

Additional Neurodevelopment Tests

Wechsler Preschool and Primary Scale of Intelligence-Third Edition (WPPSI-III)

The WPPSI-III is an intelligence test administered to children from 2 years, 6 months to 7 years, 3 months. Two forms cover the age range: 1) 2 years, 6 months - 3 years, 11 months 2) 4 years, zero months – 7 years, 3 months. The form used in the Tar Creek cohort is the 4 years, zero months – 7 years, 3 months (notated 4:0-7:3). The test takes 45 minutes to administer to the child. The test has subtests that measure verbal IQ (VIQ), performance IQ (PIQ), full-scale IQ (FSIQ), general language composite (GLC) and processing speed quotient (PSQ). Wechsler tests are the most widely used tests of intelligence, which allows for direct comparison among studies (Isaacs and Oates, 2008).

Covariates

Maternal IQ and home quality scores using the Home Observation for Measurement of the Environment inventory (HOME) are explored and potential confounders or covariates based on previous studies (Belfort, 2016) . HOME evaluates the ability of the home environment to meet the cognitive and emotional needs of a child (Frankenburg, 1986). HOME scores were divided into cognitive (CHS) and emotional subscales (EHS) with higher scores indicating home environments that support neurodevelopment. HOME scores were assessed on the same visit as the BRIEF and BASC-2 assessments.

Statistical Analysis

We used SAS 9.3 statistical software for data analysis. The primary analysis seeks to investigate the association between prenatal metals exposure (arsenic, lead and manganese) with BRIEF and BASC-2 scores. We modeled all metal concentrations and outcomes scores as

continuous variables. Each metal and subdomain was modeled separately, along with covariates. Metal values were mean-centered and log-transformed in the models.

Only 52 subjects reported HOME scores, therefore we used multiple imputations to generate reasonable values for the missing HOME scores. We conducted sequential monotone imputation. The first model included all continuous variables. We then added categorical variables one at a time from least to most missingness. We included all metal concentrations, and covariates as well as additional variables to help predict missing HOME scores. We imputed 10 datasets then conducted our analysis among the 10 full datasets using PROC MI ANALYZE. The imputation dataset was limited to children with complete outcome information.

The final multivariate model includes covariates found to be significant as well as other covariates based on previous literature such including maternal education and smoking. At the time of assessment, some children were older than the 5 years and 11 months. Since the questionnaires are normalized for this age and younger, we analyzed and reported raw scores for the BASC-2 and BRIEF tests. We adjusted for age our models.

RESULTS

Table 1.1 shows the study population characteristics. The mean (SD) age of the children at the time of assessment was 78.7 (6.45) months or 6.6 years of age. 48.8% of the children tested were girls. Mean (SD) cognitive and emotional HOME scores were 7.06 (1.08) and 5.61 (1.43) respectively.

Mean (SD) maternal age at the time of birth was 25.0 (6.29). Mean (SD) maternal IQ was 102.7 (17.0). The majority of mothers (56.7%) reported living with a partner. 60.6% of mothers were employed. 19.3% of mothers reported an annual household income of less than $10,000. 17.5% of mothers reported an annual household income between $10,000 and $20,000. 36.8% of

mothers reported an annual household income between $20,000 and $40,000. 26.3% of mothers reported an annual household income over $40,000. 25% of mothers had less than a high school education. 43.3% had at least a high school diploma or vocational training. 31.7% of mothers had some college education or beyond.

Table 1.1 Characteristics of Child Neurobehavioral Study Population at Tar Creek

Variable	Sample size (n)	Mean (SD) or proportion (%)
Child age (months)	122	78.7 (6.45)
Child gender = female	121	48.8
Cognitive HOME Score	50	7.06 (1.08)
Emotional HOME Score	51	5.61 (1.43)
Mothers age at birth	105	25.0 (6.29)
Maternal IQ	98	102.7 (17.02)
Married or living with a partner	104	56.7
Employed	104	60.6
Household income	57	
Less than $10,000		19.3
$10,000 to $20,000		17.5
$20,000 to $40,000		36.8
Greater than $40,000		26.3
Maternal education	104	
Less than high school education		25
High school diploma or vocational		43.3
Some college and/or beyond		31.7

As mentioned previously, blood metal concentrations were fairly low (Table 1.2). The mean (SD) cord blood lead was 0.529 (0.386) µg/dL. The mean (SD) arsenic and manganese concentrations were 0.274 (0.115) µg/dL and 3.86 (1.32) µg/dL respectively. The distribution of metals revealed a skewed distribution. Median lead, arsenic and manganese concentrations were 0.457 µg/dL, 0.24 µg/dL and 4.00 µg/dL respectively. The range of cord blood concentrations for lead, arsenic and manganese were 0.01-3.14 µg/dL, 0.04-0.54 µg/dL and 0.901-8.02 µg/dL respectively.

Table 1.2. Distribution of Metals Concentrations in Cord blood in Tar Creek Cohort

Metal	N	Mean (SD)	P25	Median	P90	Min	Max
Lead	122	0.529 (0.386)	0.307	0.457	0.949	0.01	3.14
Arsenic	118	0.274 (0.115)	0.20	0.24	0.43	0.04	0.54
Manganese	122	3.86 (1.32)	2.82	4.00	5.38	0.901	8.02

Units are µg/dL

Metals concentrations differed by gender (Table 1.3). Girls had higher cord blood lead with a mean of 0.545 (0.491) µg/dL while the mean level for boys was 0.514 (0.245) µg/dL. The difference between blood levels for girls and boys was statistically significant with a p-value of 0.009. For cord blood manganese, boys had significantly higher levels with a mean of 4.02 (1.37) µg/dL. The mean level for girls was 3.69 (1.25) µg/dL. The difference was statistically significant with a p-value <0.0001. No significant sex difference was found for cord-blood arsenic levels.

Table 1.3 Distribution of Metals Exposure in Cord-blood at Stratified by Gender

Lead			
	Girls	**Boys**	**P-value**
N	60	62	
Mean (SD)	0.545 (0.491)	0.514 (0.245)	0.009
Median	0.452	0.48	
Min	0.01	0.027	
Max	3.14	1.16	

Arsenic			
	Girls	**Boys**	**P-value**
N	58	60	
Mean (SD)	0.274 (0.127)	0.274 (0.103)	0.614
Median	0.275	0.255	
Min	0.04	0.06	
Max	0.54	0.53	

Manganese			
	Girls	**Boys**	**P-value**
N	60	62	
Mean (SD)	3.69 (1.25)	4.02 (1.37)	<0.0001
Median	3.69	4.2	
Min	0.901	1.29	
Max	7.59	8.02	

BRIEF

Table 1.4 shows the distribution of BRIEF raw scores. The mean (SD) BRIEF raw general composite scores (GEC) was 94 (26). The mean (SD) raw scores for the sub-domains were 39 (12.0) for Inhibitory Self-Control Index (ISCI), 28 (8.24) for Flexibility Index (FI) and the 41 (11.6) for the Emergent Metacognition Index (EMI). The median GEC, ISCI, FI and EMI raw scores were 87, 36, 25 and 38 respectively. The range of GEC, ISCI, FI and EMI raw scores were 63-168, 26-73, 20-55 and 27-76 respectively.

Table 1.4 Neurobehavioral Outcomes – BRIEF Raw Scores

BRIEF (N=121)	Mean (SD)	Median	Min	Max
Inhibitory Self-Control Index	39 (12)	36	26	73
Flexibility Index	28 (8.24)	25	20	55
Emergent Metacognition Index	41 (11.6)	38	27	76
General Composite Score	94 (26)	87	63	168

We did not find a significant association between raw BRIEF scores and lead in our multivariable models (Table 1.5). The β (SE) for lead in the ISCI model was 0.283 (1.40) with a p-value of 0.839. The β (SE) for lead in the FI model was 0.009 (1.05) with a p-value of 0.993. The β (SE) for lead in the EMI model was 0.679 (1.04) with a p-value of 0.512. Finally, the β (SE) for lead in the GEC model was 0.882 (2.69) with a p-value of 0.743.

Maternal IQ was significantly associated with the Flexibility Index (FI) with β (SE) = -0.086 (0.037), p = 0.019. Maternal IQ was not associated with ISCI, EMI or GEC scores with β (SE) = 0.061 (0.047), p = 0.192, β (SE) = -0.027 (0.054), p = 0.615 and β (SE) = -0.149 (0.112), p = 0.183 respectively. We also found a significant association between gender and ISCI score with β (SE) = 5.16 (1.92), p= 0.007, with boys having higher scores than girls. Gender was marginally significant for the FI subdomain and GEC with β (SE) = 2.64 (1.38), p = 0.056 and β (SE) = 7.53 (4.23), p = 0.075 respectively. Gender was not significantly associated with EMI with β (SE) = 1.16 (1.91), p = 0.544. Though child age was negatively associated with ISCI, FI, EMI and GEC raw scores, none of the associations were significant with p-values of 0.701, 0.373, 0.136 and 0.310 respectively.

Cognitive HOME score was negatively associated with Global Executive Composite scores with β (SE) = -6.12 (2.11), p-value 0.004. The same significant association was found with the ISCI and EMI domains with β (SE) = -2.63 (0.990), p= 0.008 and β (SE) = -3.29 (0.977), p = 0.001 respectively. Cognitive HOME score was marginally associated with FI with β (SE) = -1.16 (0.643), p = 0.071. Though emotional HOME scores were also negatively associated with ISCI, FI, EMI and GEC raw scores, none of the associations were significant with p-values of 0.420, 0.257, 0.195 and 0.266 respectively.

Table 1.5 Association between Lead and BRIEF using Multivariable Regression

Variable	Inhibitory Self-Control Index	Flexibility Index	Emergent Metacognition Index	Global Executive Composite
Lead				
β (SE)	0.283 (1.40)	0.009 (1.05)	0.679 (1.04)	0.882 (2.69)
p-value	0.839	0.993	0.512	0.743
Maternal IQ				
β (SE)	-0.061 (0.047)	-0.086 (0.037)	-0.027 (0.054)	-0.149 (0.112)
p-value	0.192	**0.019**	0.615	0.183
Child age (years)				
β (SE)	-0.747 (1.94)	-1.25 (1.41)	-2.98 (2.00)	-4.43 (4.36)
p-value	0.701	0.373	0.136	0.310
Gender				
β (SE)	5.16 (1.92)	2.64 (1.38)	1.16 (1.91)	7.53 (4.23)
p-value	**0.007**	0.056	0.544	0.075
Cognitive HOME score				
β (SE)	-2.63 (0.990)	-1.16 (0.643)	-3.29 (0.977)	-6.12 (2.11)
p-value	**0.008**	0.071	**0.001**	**0.004**
Emotional HOME score				
β (SE)	-0.606 (0.753)	-0.565 (0.499)	-0.843 (0.650)	-1.70 (1.53)
p-value	0.420	0.257	0.195	0.266

We did not find a significant association between raw BRIEF scores and arsenic in our multivariable models (Table 1.6). The β (SE) for arsenic in the ISCI model was -2.86 (2.31) with a p-value of 0.216. The β (SE) for arsenic in the FI model was -0.774 (1.69) with a p-value of 0.647. The β (SE) for arsenic in the EMI model was -3.44 (2.52) with a p-value of 0.174. Finally, the β (SE) for arsenic in the GEC model was -6.76 (5.51) with a p-value of 0.219.

Maternal IQ was marginally associated with the Flexibility Index (FI) with β (SE) = -0.071 (0.039), p = 0.064. Maternal IQ was not associated with ISCI, EMI or GEC scores with β (SE) = -0.024 (0.048), p = 0.613, β (SE) = 0.006 (0.054), p = 0.913 and β (SE) = -0.071 (0.112), p = 0.524 respectively. We also found a significant association between gender and ISCI, FI and GEC scores with β (SE) = 5.87 (1.93), p= 0.002, β (SE) = 2.95 (1.44), p = 0.040 and β (SE) = 9.30 (4.28), p = 0.03 respectively. Again, boys had higher scores than girls. Gender was not significantly associated with EMI with β (SE) = 2.05 (1.91), p = 0.283. Though child age was negatively associated with ISCI, FI, EMI and GEC raw scores, none of the associations were significant with p-values of 0.704, 0.433, 0.190 and 0.373 respectively.

Cognitive HOME score was negatively associated with Global Executive Composite scores with β (SE) = -7.35 (2.01), p-value 0.0003. The same significant association was found with the ISCI, FI and EMI subdomains with β (SE) = -3.27 (0.952), p= 0.001, β (SE) = -1.35 (0.670), p = 0.044 and β (SE) = -3.82 (0.940), p <0.0001 respectively. Though emotional HOME scores were also negatively associated with ISCI, FI, EMI and GEC raw scores, none of the associations were significant with p-values of 0.514, 0.247, 0.268 and 0.331 respectively.

Table 1.6 Association between Arsenic and BRIEF using Multivariable Regression

Variable	Inhibitory Self-Control Index	Flexibility Index	Emergent Metacognition Index	Global Executive Composite
Arsenic				
β (SE)	-2.86 (2.31)	-0.774 (1.69)	-3.44 (2.52)	-6.76 (5.51)
p-value	0.216	0.647	0.174	0.219
Maternal IQ				
β (SE)	-0.024 (0.048)	-0.071 (0.039)	0.006 (0.054)	-0.071 (0.112)
p-value	0.613	0.064	0.913	0.524
Child age				
β (SE)	-0.747 (1.96)	-1.16 (1.48)	-2.73 (2.08)	-4.03 (4.52)
p-value	0.704	0.433	0.190	0.373
Gender				
β (SE)	5.87 (1.93)	2.95 (1.44)	2.05 (1.91)	9.30 (4.28)
p-value	**0.002**	**0.040**	0.283	**0.03**
Cognitive HOME score				
β (SE)	-3.27 (0.952)	-1.35 (0.670)	-3.82 (0.940)	-7.35 (2.01)
p-value	**0.001**	**0.044**	**<0.0001**	**0.0003**
Emotional HOME score				
β (SE)	-0.52 (0.797)	-0.606 (0.523)	-0.781 (0.705)	-1.59 (1.64)
p-value	0.514	0.247	0.268	0.331

We did not find a significant association between raw BRIEF scores and manganese in our multivariable models (Table 1.7). The β (SE) for manganese in the ISCI model was -1.07 (2.43) with a p-value of 0.659. The β (SE) for manganese in the FI model was -1.79 (1.91) with a p-value of 0.348. The β (SE) for manganese in the EMI model was -1.27 (2.50) with a p-value of 0.611. Finally, the β (SE) for manganese in the GEC model was -3.52 (5.44) with a p-value of 0.517.

Maternal IQ showed a significant, negative association with FI with β (SE) = -0.087 (0.036), p = 0.016. Maternal IQ was not significantly associated with ISCI, EMI or GEC scores with β (SE) = -0.063 (0.047), p = 0.181, β (SE) = -0.031 (0.053), p = 0.557 and β (SE) = -0.155 (0.111), p = 0.160 respectively. We also found a significant association between gender and ISCI, FI and GEC scores with β (SE) = 5.31 (1.89), p= 0.005, β (SE) = 2.81 (1.37), p = 0.040 and β (SE) = 8.01 (4.14), p = 0.053 respectively. Again, boys had higher scores than girls. Gender was not significantly associated with EMI with β (SE) = 1.38 (1.86), p = 0.467. Though child age was negatively associated with ISCI, EMI and GEC raw scores, none of the associations were significant with p-values of 0.724, 0.155 and 0.338 respectively.

Cognitive HOME score was negatively associated with Global Executive Composite scores with β (SE) = -5.83 (2.09), p-value 0.005. The same significant association was found with the ISCI and EMI domains with β (SE) = -2.54 (0.978), p= 0.009 and β (SE) = -3.16 (0.963), p = 0.001 respectively. Cognitive HOME score was not significantly associated with FI with β (SE) = -1.05 (0.655), p = 0.109. Though emotional HOME scores were also negatively associated with ISCI, FI, EMI and GEC raw scores, none of the associations were significant with p-values of 0.390, 0.175, 0.191 and 0.236 respectively.

Table 1.7 Association between Manganese and BRIEF using Multivariable Regression

Variable	Inhibitory Self-Control Index	Flexibility Index	Emergent Metacognition Index	Global Executive Composite
Manganese				
β (SE)	-1.07 (2.43)	-1.79 (1.91)	-1.27 (2.50)	-3.52 (5.44)
p-value	0.659	0.348	0.611	0.517
Maternal IQ				
β (SE)	-0.063 (0.047)	-0.087 (0.036)	-0.031 (0.053)	-0.155 (0.111)
p-value	0.181	0.016	0.557	0.160
Child age				
β (SE)	-0.695 (1.97)	0.406	-2.90 (2.04)	-4.27 (4.45)
p-value	0.724	0.330	0.155	0.338
Gender				
β (SE)	5.31 (1.89)	2.81 (1.37)	1.38 (1.86)	8.01 (4.14)
p-value	**0.005**	0.040	0.467	**0.053**
Cognitive HOME score				
β (SE)	-2.54 (0.978)	-1.05 (0.655)	-3.16 (0.963)	-5.83 (2.09)
p-value	**0.009**	0.109	**0.001**	**0.005**
Emotional HOME score				
β (SE)	-0.661 (0.769)	-0.671 (0.495)	-0.896 (0.685)	-1.88 (1.59)
p-value	0.390	0.175	0.191	0.236

BASC-2 Raw Scores

Table 1.8 shows the distribution of the BASC-2 raw scores for the following domains: Externalizing Problems (EP), Internalizing Problems (IP), Behavioral Symptoms Index (BSI) and Adaptive Skills (AS). The mean (SD) raw composite scores from the BASC-2 assessment were 97.0 (22.1) for Externalizing Problems, 155 (24.2) for Internalizing Problems, 298 (56.2) for Behavioral Symptoms Index and 209 (33.5) for Adaptive Skills.

The median EP, IP, BSI and AS raw scores were 92.5, 152, 289 and 212 respectively. The range of EP, IP, BSI and AS raw scores were 66 - 170, 115 - 222, 220 - 493 and 101 - 274 respectively.

Table 1.8 Neurobehavioral Outcomes - BASC-2 Raw Scores

BASC-2 (N=122)	Mean (SD)	Median	Min	Max
Externalizing Problems	97.0 (22.1)	92.5	66	170
Internalizing Problems	155 (24.2)	152	115	222
Behavioral Symptoms Index	298 (56.2)	289	220	493
Adaptive Skills	209 (33.5)	212	101	274

We found a significant, negative association between lead and Adaptive Skills with β (SE) = -5.99 (2.68), p = 0.025. Children with higher blood lead levels at birth showed worse Adaptive Skills. We did not find significant associations between lead and scores for Externalizing Problems, Internalizing Problems or the Behavior Symptoms Index. The β (SE) for lead in the EP model was 0.278 (2.36) with a p-value of 0.906. The β (SE) for lead in the IP model was 1.00 (2.80) with a p-value of 0.719. The β (SE) for lead in the BSI model was 2.73 (5.83) with a p-value of 0.639.

Maternal IQ was significantly associated with raw AS scores with β (SE) = 0.401 (0.122), p = 0.001. Maternal IQ was not associated with EP, IP or BSI scores with β (SE) = 0.0008 (0.091), p = 0.993, β (SE) = 0.11 (0.116), p = 0.342 and β (SE) = -0.083(0.22), p = 0.705 respectively. We also found a significant association between gender and EP scores with β (SE) = 8.40 (3.64), p= 0.021. Boys had higher scores than girls. Gender was marginally associated with AS scores with β (SE) = -8.72 (4.85), p = 0.072. Boys had lower AS scores. We did not find significant associations between gender and raw IP and BSI scores with β (SE) = -5.80 (4.21), p = 0.168 and β (SE) = 9.64 (8.89), p = 0.278 respectively. We found a significant association between Adaptive Skills and age. Older children had higher AS scores with β (SE) = 12.4 (5.0), p = 0.013. Child age was marginally associated with BSI scores with β (SE) = -17.0 (9.10), p = 0.062. We did not find significant associations between age EP or IP with β (SE) = -4.28 (3.61), p = 0.236 and β (SE) = -4.34 (4.63), p = 0.348 respectively.

Cognitive HOME score was negatively associated with EP, IP and BSI scores with β (SE) = -7.13 (2.07), p-value 0.001, β (SE) = -5.21 (2.05), p= 0.011 and β (SE) = -23.6 (4.65), p <0.0001 respectively. Cognitive HOME score was positively associated with AS with β (SE) = 14.5 (2.21), p <0.0001. We did not find any significant associations between emotional HOME

scores and raw EP, IP, BSI or AS scores with p-values of 0.753, 0.681, 0.522 and 0.954 respectively.

Table 1.9 Association between Lead and BASC-2 using Multivariable Regression

Variable	Externalizing Problems	Internalizing Problems	Behavioral Symptoms Index	Adaptive Skills
Lead				
β (SE)	0.278 (2.36)	1.00 (2.80)	2.73 (5.83)	-5.99 (2.68)
p-value	0.906	0.719	0.639	**0.025**
Maternal IQ				
β (SE)	0.0008 (0.091)	0.11 (0.116)	-0.083(0.22)	0.401 (0.122)
p-value	0.993	0.342	0.705	**0.001**
Child age (years)				
β (SE)	-4.28 (3.61)	-4.34 (4.63)	-17.0 (9.10)	12.4 (5.0)
p-value	0.236	0.348	0.062	**0.013**
Gender				
β (SE)	8.40 (3.64)	-5.80 (4.21)	9.64 (8.89)	-8.72 (4.85)
p-value	**0.021**	0.168	0.278	0.072
Cognitive HOME score				
β (SE)	-7.13 (2.07)	-5.21 (2.05)	-23.6 (4.65)	14.5 (2.21)
p-value	**0.001**	**0.011**	**<0.0001**	**< 0.0001**
Emotional HOME score				
β (SE)	0.365 (1.16)	-0.646 (1.57)	-1.90 (2.97)	-0.092 (1.60)
p-value	0.753	0.681	0.522	0.954

Arsenic was marginally associated with lower Externalizing Problems with β (SE) = -7.18 (4.21) and a p-value of 0.089 (Table 1.9). We did not find significant associations between arsenic and raw scores for Internalizing Problems, Behavior Symptoms Index or Adaptive Skills. The β (SE) for arsenic in the IP model was -1.37 (3.89) with a p-value of 0.725. The β (SE) for arsenic in the BSI model was -14.2 (9.67) with a p-value of 0.143. The β (SE) for arsenic in the AS model was 1.86 (4.43) with a p-value of 0.979.

In our arsenic model, maternal IQ was significantly associated with raw AS scores with β (SE) = 0.387 (0.137), p = 0.005. Maternal IQ was not associated with EP, IP or BSI scores with β (SE) = 0.091 (0.090), p = 0.311, β (SE) = 0.132 (0.119), p = 0.266 and β (SE) = 0.064 (0.229), p = 0.781 respectively. We also found a significant association between gender and EP scores with β (SE) = 9.65 (3.60), p= 0.007. Boys had higher scores than girls. Gender was also significantly associated with AS scores with β (SE) = -10.6 (5.01), p = 0.034. Boys had lower AS scores. We did not find significant associations between gender and raw IP and BSI scores with β (SE) = -5.19 (4.33), p = 0.231 and β (SE) = 11.5 (9.05), p = 0.203 respectively. We found a significant association between age and Adaptive Skills. Older children had higher AS scores with β (SE) = 11.6 (5.42), p = 0.032. Older child also had significantly lower BSI scores with β (SE) = -17.8 (9.15), p = 0.052. We did not find significant associations between age EP or IP with β (SE) = -5.08 (3.34), p = 0.128 and β (SE) = -4.62 (4.89), p = 0.344 respectively.

Cognitive HOME score was negatively associated with EP, IP and BSI scores with β (SE) = -8.52 (1.97), p < 0.0001, β (SE) = -5.49 (2.10), p= 0.009 and β (SE) = -25.8 (4.54), p <0.0001 respectively. Cognitive HOME score was positively associated with AS with β (SE) = 15.0 (2.27), p <0.0001. We did not find any significant associations between emotional HOME

scores and raw EP, IP, BSI or AS scores with p-values of 0.626, 0.675, 0.641 and 0.894 respectively.

Table 1.10 Association between Arsenic and BASC-2 using Multivariable Regression

Variable	Externalizing Problems	Internalizing Problems	Behavioral Symptoms Index	Adaptive Skills
Arsenic				
β (SE)	-7.18 (4.21)	-1.37 (3.89)	-14.2 (9.67)	1.86 (4.43)
p-value	0.089	0.725	0.143	0.979
Maternal IQ				
β (SE)	0.091 (0.090)	0.132 (0.119)	0.064 (0.229)	0.387 (0.137)
p-value	0.311	0.266	0.781	**0.005**
Child age				
β (SE)	-5.08 (3.34)	-4.62 (4.89)	-17.8 (9.15)	11.6 (5.42)
p-value	0.128	0.344	**0.052**	**0.032**
Gender				
β (SE)	9.65 (3.60)	-5.19 (4.33)	11.5 (9.05)	-10.6 (5.01)
p-value	**0.007**	0.231	0.203	**0.034**
Cognitive HOME score				
β (SE)	-8.52 (1.97)	-5.49 (2.10)	-25.8 (4.54)	15.0 (2.27)
p-value	**<0.0001**	**0.009**	**<0.0001**	**< 0.0001**
Emotional HOME score				
β (SE)	0.540 (1.11)	-0.665 (1.59)	-1.36 (2.92)	-0.220 (1.64)
p-value	0.626	0.675	0.641	0.894

Manganese was marginally associated with lower with Adaptive Skills with β (SE) = -9.88 (5.74) and a p-value of 0.085 (Table 1.10). We did not find significant associations between manganese and raw scores for Externalizing Problems, Internalizing Problems and Behavior Symptoms Index. The β (SE) for manganese in the EP model was 8.02 (5.26) with a p-value of 0.128. The β (SE) for manganese in the IP model was 2.70 (5.42) with a p-value of 0.619. The β (SE) for manganese in the BSI model was 13.3 (12.1) with a p-value of 0.272.

In our manganese model, maternal IQ was significantly associated with raw AS scores with β (SE) = 0.437 (0.132), p = 0.001. Maternal IQ was not associated with EP, IP or BSI scores with β (SE) = -0.010 (0.092), p = 0.910, β (SE) = 0.103 (0.117), p = 0.380 and β (SE) = -0.111 (0.228), p = 0.627 respectively.

We also found a significant association between gender and EP scores with β (SE) = 7.48 (3.58), p= 0.037. Boys had higher scores than girls. Gender was marginally associated with lower AS scores with β (SE) = -8.25 (4.77), p = 0.084. We did not find significant associations between gender and raw IP and BSI scores with β (SE) = -6.00 (4.19), p = 0.152 and β (SE) = 8.38 (8.89), p = 0.346 respectively. We found a significant association between age and Adaptive Skills. Older children had higher AS scores with β (SE) = 12.5 (4.82), p = 0.009. Older children also had significantly higher BSI scores with β (SE) = 17.4 (8.80), p = 0.048. We did not find significant associations between age EP or IP with β (SE) = -4.57 (3.45), p = 0.185 and β (SE) = -4.40 (4.51), p = 0.330 respectively.

Cognitive HOME score was negatively associated with EP, IP and BSI scores with β (SE) = -7.85 (2.06), p = 0.0001, β (SE) = -5.45 (2.14), p= 0.011 and β (SE) = -24.8 (4.82), p <0.0001 respectively. Cognitive HOME score was positively associated with AS with β (SE) = 15.4 (2.30), p <0.0001. We did not find any significant associations between emotional HOME

scores and raw EP, IP, BSI or AS scores with p-values of 0.440, 0.801, 0.763 and 0.524 respectively.

Table 1.11 Association between Manganese and BASC-2 using Multivariable Regression

Variable	Externalizing Problems	Internalizing Problems	Behavioral Symptoms Index	Adaptive Skills
Manganese				
β (SE)	8.02 (5.26)	2.70 (5.42)	13.3 (12.1)	-9.88 (5.74)
p-value	0.128	0.619	0.272	0.085
Maternal IQ				
β (SE)	-0.010 (0.092)	0.103 (0.117)	-0.111 (0.228)	0.437 (0.132)
p-value	0.910	0.380	0.627	**0.001**
Child age				
β (SE)	-4.57 (3.45)	-4.40 (4.51)	17.4 (8.80)	12.5 (4.82)
p-value	0.185	0.330	**0.048**	**0.009**
Gender				
β (SE)	7.48 (3.58)	-6.00 (4.19)	8.38 (8.89)	-8.25 (4.77)
p-value	**0.037**	0.152	0.346	0.084
Cognitive HOME score				
β (SE)	-7.85 (2.06)	-5.45 (2.14)	-24.8 (4.82)	15.4 (2.30)
p-value	**0.0001**	**0.011**	**<0.0001**	**< 0.0001**
Emotional HOME score				
β (SE)	0.877 (1.13)	-0.422 (1.67)	-0.921 (3.06)	-1.04 (1.64)
p-value	0.440	0.801	0.763	0.524

DISCUSSION

We sought to provide further insight into the neurodevelopmental outcomes in the Tar Creek birth cohort MATCH study. Here, we focus on neurobehavioral outcomes assessed at follow-up in children 5-7 years of age. General measures of behavior including emotional, social and executive functioning were assessed using BRIEF and BASC-2 tests.

In our BRIEF analysis, we generally found that higher blood lead was associated with poorer executive function though the associations were not significantly significant. However, our analysis showed a negative association between executive function and concentrations of arsenic and manganese. This could be due to residual confounding. In addition, the levels of metals exposures are relatively low. Any effect of arsenic could be masked by the lead, which is known to be detrimental to neurodevelopment even at low levels. Though manganese showed the same relationship as arsenic, the fact that manganese is an essential nutrient further complicates the relationship. Other studies have found U –shaped relationship with manganese. Our analyses of covariates were consistent with expectations. We found that children exhibited better executive function with increasing cognitive HOME scores. This association was significant. Although we observed the same trend with emotional HOME scores, there was no statistical significance. Additionally, boys consistently showed poorer executive function when adjusting for metals, age, maternal IQ and HOME scores. This association was significant in many of our analyses.

During our analysis of emotional and social functioning using the BASC-2 domains, we found an association that increased blood lead levels at birth was associated with poorer adaptive skills in children 5 to 7 years of age. Again, our analysis showed a negative association between BASC-2 sub-domains with the exception of adaptive skills. These observations are counter-

intuitive, once again revealing that residual confounding and masked effects of lead may be at play. The relationship between manganese and the BASC-2 domains were consistent with lead; however, none of the associations were significant. The discrepancy in directionality of the association between BRIEF and BASC-2 suggests a complex association between manganese and neurobehavioral outcomes that may need to be teased apart by further investigating behavioral sub-domains.

Cognitive HOME scores were significantly associated with BASC-2 scores in all our sub-domain models. The direction of association was consistent with expectations. We found Externalizing Problems, Internalizing Problems and the Behavioral Symptoms Index composite scores were lower with increasing cognitive HOME scores. Cognitive HOME scores were positively associated with better adaptive skills. In addition, we consistently observed statistically significant higher Externalizing Problems in boys across all models.

A major limitation of the study is the loss to follow-up (LTFU). While the baseline cohort contained 713 mother-infant pairs, only 122 BASC-2 and 121 BRIEF scores were available for our analysis. Loss to follow-up could be due to various factors. Multiple imputations dataset should accurately reflect missing data assuming the data is missing at random. LTFU severely limited our sample size, and did not allow us to take advantage of the repeated measures of exposure.

Generalizability is limited due to the characteristics of the study population. The major of participants identify as Native American or white. For this reason, we did not include measures of race in our analysis, allowing us to preserve statistical power given our limited sample size. In addition, high economic and financial instability results in high population instability. Many of the residents became increasing aware of contamination issues surrounding the superfund site.

Another limitation of our study is that metals exposure was measured using cord blood at birth as a biomarker. We obtained, but did not utilize repeated measures of exposure from other biomarkers such as hair and nail due to low sample size. Unfortunately, we did not obtain repeated measures of blood metal levels for analysis. While levels of metals in cord blood provides insight into the role of prenatal exposure to later neurobehavioral outcomes, other biomarkers, particularly hair and nail, may provide better insight into continued metal exposure during the early years of life. While we did not observe metals interactions, again this may be due to the limited study size.

We did not include child IQ in our models. Future investigations should explore child IQ as both a covariate and outcome. Additionally, measures of maternal depression are also available for this study population. Future analysis will investigate the association between depression in mothers and neurodevelopment in children. Future studies will also investigate the association between metals and other neurocognitive outcomes such memory, visual-motor skills and attention using the Wide Range Assessment of Memory and Learning (WRAML), Wide Range Assessment of Visual Motor Ability (WRAVMA) and Conners' Kiddie Continuous Performance Test (K-CPT).

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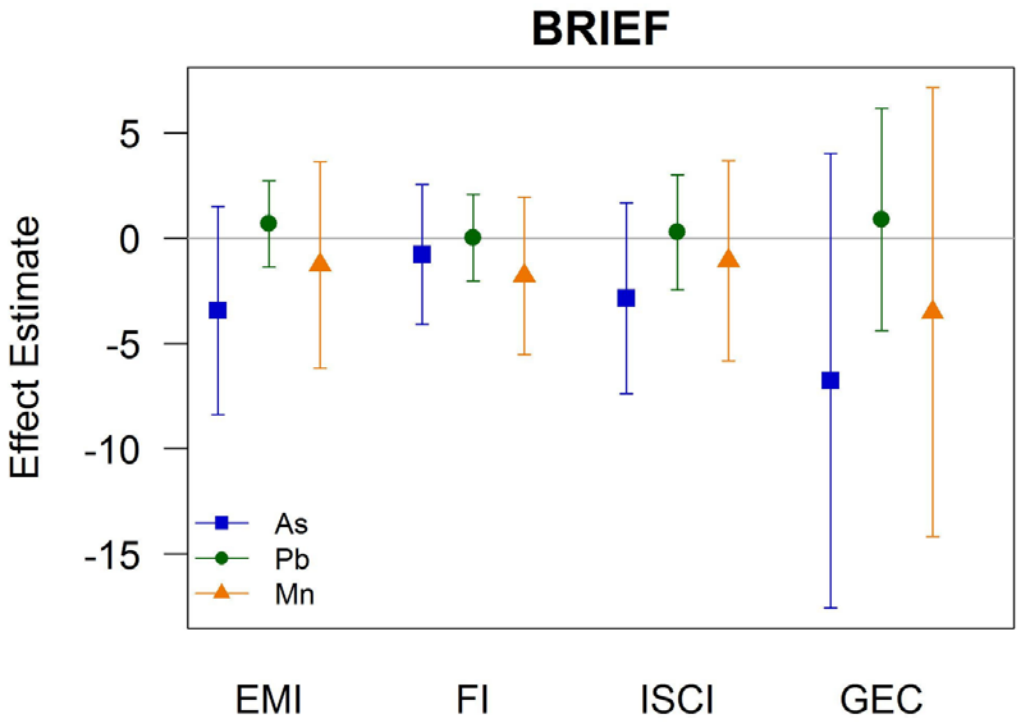
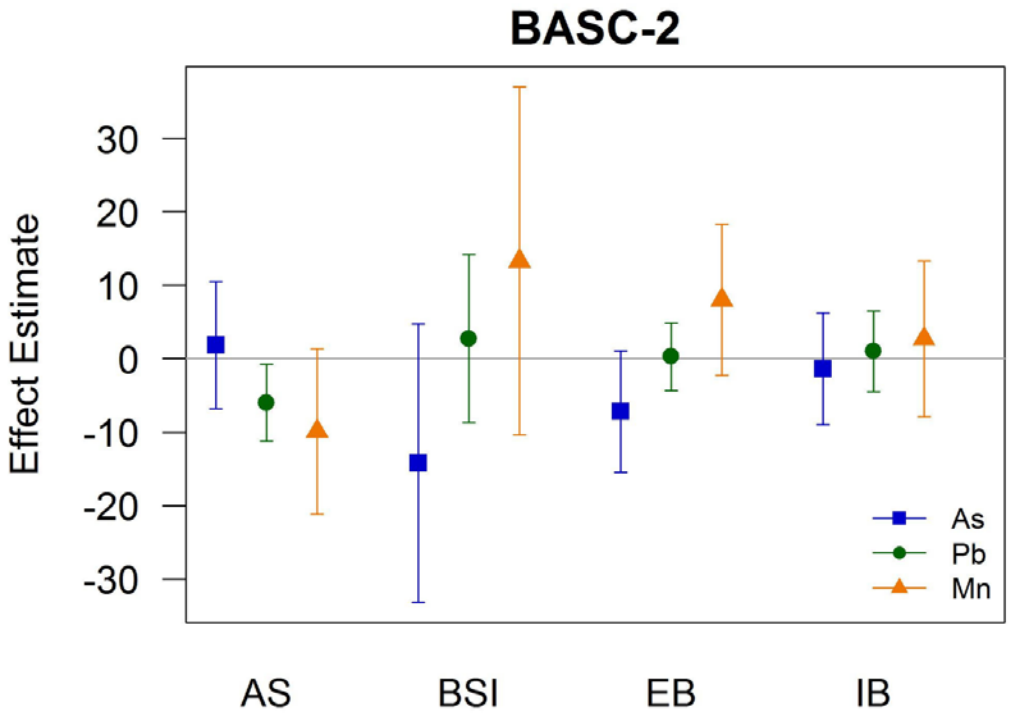
Figure 1.1 Effect Estimates of Arsenic, Lead and Manganese for BRIEF domains



EMI = Adaptive Skills
FI = Behavior Symptoms Index
ISCI = Externalizing Behavior
GEC = Internalizing Behavior

Figure 1.2 Effect Estimates of Arsenic, Lead and Manganese for BASC-2 domains



AS = Adaptive Skills
BSI = Behavior Symptoms Index
EB = Externalizing Behavior
IB = Internalizing Behavior

CHAPTER III

Exposure to Arsenic, Lead and Manganese and the Association with Postpartum Depression in Mothers from the Tar Creek Cohort

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ABSTRACT

Background: Postpartum depression affects 10-15% of new mothers. There is increasing evidence that exposure to metals may play a role in the development of depression.

Objective: This study investigates the association between arsenic, lead and manganese and postpartum depression in the Tar Creek cohort.

Design: We conducted a cross-sectional analysis of mothers in the Tar Creek Superfund Cohort Study. We measured maternal metal concentrations in blood at the time of delivery. Mothers were administered the Edinburgh Postnatal Depression Scale (EPDS) within hours after delivery.

Results: Results of a median regression analysis indicate that mothers with increased arsenic concentration were more likely to suffer from PPD, β (SE) = 1.03 (0.45). The association was statistically significant ($p = 0.01$) when adjusting for lead and manganese exposure, maternal age, maternal education, household income, smoking status, living with a partner and history of depression. We also found a significant interaction between lead and arsenic ($\beta = 1.13$, $p=0.017$). No other interactions between arsenic, lead and manganese were significant.

Conclusion: Our study provides further evidence that heavy metals play an important role in depression etiology. Increased arsenic exposure is positively associated with postpartum depression in mothers from the Tar Creek cohort. In addition, we found a significant interaction between arsenic and lead. This further highlights the necessity of taking into account metal co-exposures when investigating neurological health outcomes such as PPD.

INTRODUCTION

Postpartum Depression

Mental health is a major public health concern. Recent events of violence such mass shooting and celebrity suicides have increased public outcry for better mental health resources.

In the United States alone, depression affects over 20 million people (NIMH) Postpartum depression (PDD) affects 10-15% of new mothers (O'Hara, 1996). Postpartum is an especially vulnerable time for new mothers and their infants.

Rapid changes in estrogen, progesterone and cortisol hormones (within 48 hours of delivery) are believed to be the cause of PPD. However, the exact cause remains unknown. Risk factors for PPD include younger age, lack of social support, paternal history of depression, major life events and marital status (Patel, 2012). However, the most common risk factor for PPD is a history of depression. The onset of PPD is within 4 weeks of delivery and can last into the first postnatal year.

The most common screening tool for postnatal depression is the Edinburgh Postnatal Depression Scale (EPDS) developed by Cox et al in 1987. Diagnosis of postnatal depression (considered a subcategory of major depressive disorder) utilizes the Diagnostic Statistical Manual of Mental Disorder, 4th edition (DSM-IV) criteria. Mothers receive treatment for PDD through a variety of methods including psychotherapy and pharmacotherapy. Psychological counseling is often the first course of actions due to maternal concerns in the use of medication while breastfeeding.

Metals and Mental Health

Metals such as arsenic, lead and manganese are readily found in the earth's crust. Exposure primarily occurs through contamination of drinking water, direct exposure to soil, and inhalation of house dust. While occupational studies have focused on high-level exposure, numerous studies have shown that these metals are neurotoxic even at low levels. Increasingly, investigations are focusing on the association between heavy metal exposures and neuropsychiatric outcomes.

Oxidative stress is believed to be one of the primary mechanisms of neurotoxicity for As and Mn. Metabolism of these metals produce reactive oxygen species (ROS) that can cause cellular damage, inflammation and interfere with normal cellular function (Flora, 2011 and Horning et al, 2015). Studies have also shown that both lead and manganese affect dopaminergic systems in the brain (Cory-Slechta, 1995 and Fitsanakis et al, 2006). Another important mechanism of lead neurotoxicity is the through the disruption of normal calcium function essential for many cellular processes including cell-signaling pathways (ATSDR, 2007).

The neurotoxicity of lead is well documents. Many studies have sought to investigate the impact of lead on adults. A longitudinal study of lead workers found that past exposure to lead is associated with cognitive decline in adults (Schwartz, 2001). However, in another study of NHANES data (Bouchard, 2009), investigators found that blood lead levels were associated with increased risk of mental health disorder in young adults (age 20-39). Blood lead quintiles were associated with increased major depressive disorder (p for trend = 0.05) and panic disorder (p for trend = 0.02). In addition, subjects in the highest quintile (were $\geq$ 2.11 μg/dL) had an increase in the adjusted odds ratio of 2.32 (95% CI: 1.13-4.75) for major depression and 4.94 (95% CI: 1.32-18.48) for panic disorder. A study by Eum et al, 2012 found an association between bone lead levels and depression symptoms in premenopausal or postmenopausal women who took hormone replacement therapy (HRT). Using the Metal Health Index 5-item subscale (MHI-5), women in the highest tertile of Pb concentrations reported worse depressive symptoms than those in the lowest tertile (p-trend = 0.0001). However, two studies (Liu et al, 2013 and Örün et al, 2011) specifically looking at PPD did not find an association between breast milk lead and EPDS depression scores.

Current knowledge of the effects of arsenic exposure on mental health in adults is limited but increasing. In a Mongolian study using the General Health Questionnaire (GHQ) investigators found that subjects in an arsenic-exposed village were 2.5 times as likely (95% CI: 1.1-6.0, $p = 0.037$) to experience distress as those in a arsenic-free village (Fujino et al, 2004). A cross-sectional study by Syed et al in 2012 found that arsenic-affected patients (>50 µg/L As in drinking water) in Bangladesh had significantly lower ($p = 0.002$ for males and $p < 0.001$ for females) overall quality of life (QOL) scores than non-patients. QOL scores were obtained using the World Health Organization Quality of Life – BREF assessment. The study also found that patients had significantly lower mental health status ($p < 0.001$ for males and females) than non-patients when using a self-reported questionnaire. A US study by Zierold et al (2004) found that persons whose household water arsenic concentrations are between 2 µg/L and 10 µg/L are 2.74 times as likely to report depression compared to the reference group (<2 µg/L). Mukherjee et al, 2014 investigated the association between well water arsenic and depressive symptoms in West Bengal, India. Investigators assessed symptoms using the Beck Depression Inventory, Second Edition (BDI-II). Using a score cut-off of ≥ 14 for presence of depressive symptoms, arsenic-exposed women (>10 µg/L As in drinking water) had a significantly higher prevalence of depressive symptoms ($p<0.001$) than controls.

Chronic arsenic toxicity is referred to as arsenicosis. The symptoms of arsenicosis varies and its' onset depends on the level and duration of exposure. A characteristic feature of arsenicosis is the presence of hyperpigmentation and keratosis skin lesions. Other common symptoms are weakness, cough, paresthesia, liver damage and Black foot disease (Mazumder, 2008). Symptoms of arsenicosis such as keratosis and Black foot disease are extreme and outwardly noticeable. Victims experience social and economic instability, (Keya et al, 2008),

discrimination and ostracism (Brinkel et al, 2006). Extreme physical ailments and psychosocial factors further complicate the association between arsenic toxicity and mental health.

Manganese is an essential nutrient that helps protect against oxidative stress. Ironically, Mn neurotoxicity from excess manganese may also occur through redox mechanisms. High manganese exposure has been associated with Parkinsonism symptoms (Beuter, 1994) and other neurodegenerative conditions such as manganism. Manganism initially presents itself as a psychiatric condition with symptoms such as violent behavior, emotional instability, fatigue and insomnia and often resembles Parkinson's disease. Manganism has been associated with high levels of manganese in the brain (Aschner, 2006). In a study using 2011-2012 NHANES data, investigators found that adults with depression had higher levels of urinary manganese with an adjusted OR of 1.47 (95% CI: 1.01-2.15). Information on depression was obtained using the self-reported Patient Health Questionnaire, with a cut-off of ≥ 10 categorized as having depression (Shiue et al, 2015). Another study found that male psychiatric patients in Pakistan had significantly ($p<0.001$) higher levels of hair manganese than controls (Arain et al, 2015). A study of US welders found that blood manganese was significantly associated with depression (assessed using the BDI-II). One unit increase in blood manganese concentration was associated with 1.5 times the odds of having depression (95% CI: 1.0-2.2, $p<0.05$) among the study population.

Knowledge Gap

The association between metals exposure and mental health is not well understood. Evidence exists that lead, arsenic and manganese are neurotoxic, but studies on their association with postpartum depression specifically are extremely scarce. The majority of PDD research focuses on the role of micronutrients such as iron, zinc selenium and calcium in the treatment

depression. Some studies have explored the deleterious effects of lithium treatment on neurodevelopment but the possible adverse effects other metals are primarily ignored. Our study seeks to contribute to our understanding of how arsenic, manganese and lead, even at low-level exposures, may play a role in the etiology of postpartum depression.

METHODS

Study Design and Population

Study design and population have been described previously (Ettinger et al., 2009; Zota et al., 2009a). Briefly, the Tar Creek Superfund site is a former mining site located in northeast Oklahoma. In 1983, the EPA added the site to the National Priority List due to extensive metal contamination of water and soil. Approximately 30,000 people live in the area with some residents being of Native American descent.

The Tar Creek study is a prospective birth cohort to study biological markers of early life exposure to environmental toxicants and health outcomes. Data collected includes metals exposure, stress indicators, infant growth and child development. Subjects recruited were pregnant women giving birth at Integris Hospital in Miami, Oklahoma who planned to live in the area for the next two years. Information on sociodemographic characteristics, potential sources of exposures and psychosocial stress is collected using interviewer-administered questionnaires. Maternal blood samples and cord blood samples were collected within a 12-hour window of delivery birth. The study collected data for 713 mother-infant pairs at birth. Mental health indicators were measured every six months from baseline to two years. Study participants are predominantly white and Native American population.

Exposure Assessment

Maternal whole blood samples were collected at delivery. The HSPH Trace Metals Laboratory analyzed arsenic, lead and manganese levels using inductively coupled plasma mass spectrometry (ICP-MS). The limit of detection for whole blood arsenic, manganese and lead using this technique is 0.02 μg/dL. Quality control measures include external calibration with standard reference materials, use of an indium internal standard and analysis of replicates. The measurements for 60 subjects we excluded from the analysis due to instrumental error.

Screening Tools for Maternal Mental Health

Edinburgh Postnatal Depression Scale (EPDS)

The EPDS is a self-administered questionnaire, used as a screening tool for depression. The EPDS is a 10-question survey scored on a Likert scale. The questionnaire asked respondents to indicate how they have felt over the past 7 days. The maximum score is 30. Participants with scores ≥ 10 may possibly have depression. Respondents whose scores are ≥ 13 are likely to be suffering from depression (Wisner et al, 2002). Investigators administer an initial questionnaire at enrollment (within hours of delivery) Investigators administer follow-up surveys over the phone or in person at months 3, 6, 9, 12, 15, 18, 21 and 24.

Statistical Analysis

Arsenic, lead and manganese concentrations were log-transformed and modeled continuously. We also mean centered our metals concentrations. Depression scores are modeled continuously from 0 to 30. Due to the non-normal distribution of depression scores, the associations between depression and metal concentrations were analyzed using quantile regression (modeling based on the median rather than the mean). We modeled the association at several quantiles: 25^{th}, 30^{th}, 40^{th}, 50^{th} (median), 60^{th}, 70^{th}, 80^{th} and 90^{th}.

Preliminary analyses revealed household income is a significant covariate. However, only 66% of subjects in the analytical data reported income data. Therefore, we performed multiple imputations in order to include income as a covariate in the multivariable model. We excluded mothers who were missing depression scores from the imputation dataset. We first imputed a dataset containing only continuous predictors. We then conducted sequential monotone logistic imputations in which we added categorical imputation variables one at a time from least to most missing. Covariates of interest are age, maternal and paternal education, alcohol use, smoking, income and employment. We imputed ten dataset iterations. The final analytical dataset included 582 mothers. We used the non-imputed dataset for our univariate models.

We fit a quantile regression model to investigate associations between predictor variables and depression. Some of the covariates used in the final model e.g. age, were chosen *a priori* will were selected based on the results of our univariate analyses. We analyzed our data using SAS 9.4.

RESULTS

Table 2.1 describes characteristics of the analytical study population (n = 582). Mean (SD) maternal age at birth was 24.6 (5.5) years. 35.7% of mothers reported being a current smoker. 22.8% of mothers reported drinking alcohol at least one day in a week over the last five years. 15.8 % of mothers had a maternal history of depression (reported previous prescription medication for depression). Most mothers (65.7%) were married or lived with a partner. 58.6% of mothers were unemployed. This includes mothers not looking for employment. 23.5% of mothers reported an annual household income of less than $10,000. 24.8% reported and annual household income between $10,000 and $20,000. 33.3% had household incomes between $20,000 and $40,000. 18.4% reported annual household incomes of over $40,000. 25.8 % of

mothers did not complete high school or vocational training. 42.7% of mothers had at least a high school diploma or vocational training. 31.5% had some college education or beyond. Respondents reported that 21.9% of fathers had less than high school education, 45.0% had at least a high school diploma or vocational training, 33.1% of fathers had at least some college education or beyond.

Table 2.1 Maternal Characteristics in Tar Creek Cohort

Variable	Sample size (n)	Mean (SD) or proportion (%)
Mothers age at birth	581	24.6 (5.5)
Current smoker	582	35.7
Drank 1 or more days in a week in the last 5 years	518	22.8
Doctor prescribed medication for depression	575	15.8
Married or living with a partner	565	65.7
Unemployed	575	58.6
Household income	387	-
Less than $10,000	91	23.5
$10,000 to $20,000	96	24.8
$20,000 to $40,000	129	33.3
Greater than $40,000	71	18.4
Maternal education	581	-
Less than high school education	150	25.8
High school diploma or vocational	248	42.7
Some college and/or beyond	183	31.5
Paternal education	242	-
Less than high school education	53	21.9
High school diploma or vocational	109	45.0
Some college and/or beyond	80	33.1

Table 2.2 shows the distribution of maternal blood arsenic, lead and manganese at delivery. The mean (SD) blood arsenic, manganese and lead levels at delivery were 0.188 (0.167) µg/dL, 2.55 (1.05) µg/dL and 0.713 (0.437) µg/dL respectively. The results indicate the distributions of blood metal concentration are highly skewed thus making it appropriate to log-transform them in our statistical models. The median arsenic, manganese and lead levels were 0.144 µg/dL, 2.32 µg/dL and 0.601 µg/dL respectively. The range of arsenic concentration was 0.023 – 2.41 µg/dL. The range of manganese levels was 0.802 – 11.74 µg/dL. The range of lead concentrations was 0.027 – 3.14 µg/dL.

Table 2.2 also shows the summary statistics for EPDS scores. We had depression scores for 582 mothers at delivery. The mean (SD) EPDS score was 5.19 (5.50) with a range of 0 to 27. The median EPDS score was 4.0. EPDS scores were not normally distributed. We therefore, as stated in the methods, choose to conduct quantile regression in our models.

Table 2.2 Maternal Blood Concentrations and EPDS Scores at Delivery

Variable	N	Mean (SD)	25th pct	Median	75th pct	Min	Max
Arsenic (ug/dL)	578	0.188 (0.167)	0.097	0.144	0.220	0.023	2.41
Manganese (ug/dL)	580	2.55 (1.05)	1.91	2.32	2.98	0.802	11.74
Lead (ug/dL)	580	0.713 (0.437)	0.410	0.601	0.898	0.027	3.14
EPDS Score	582	5.19 (5.50)	0.0	4.0	8.0	0.0	27.0

Table 2.3 shows the results of univariate regression at the 50th quantile using the imputed data. We found a positive association between blood arsenic and maternal depression with, β (SE) = 1.17 (0.60) but the results were only marginally significant (p = 0.064). Manganese and lead were not significantly associated with depression in our univariate analysis. Higher maternal age at birth was marginally associated with lower depression scores, β (SE) = - 0.105 (0.058), p = 0.07. We found an association between maternal education and EPDS scores (p=0.001). Mothers with at least a high school diploma or vocational training had lower scores than mothers who did not complete such training with β (SE) = -3.0 (0.80), p = 0.0002. Mothers with some college education also had less depression compared to mothers who did not complete high school with β (SE) = -3.0 (1.05), p = 0.005. Household income was also a significant predictor of PPD. Overall, mothers whose annual household income was > $10,000 had significantly lower scores (p for trend = 0.039). Mothers whose annual income was between $20,001 and $40,000 had significantly lower EPDS scores with β (SE) = -3.0 (1.04), p = 0.004 compared to those whose annual household income was less than $10,000. We did not find significant associations for mothers with household income between $10,000 to $20,000 and over $40,000 with β (SE) = -2.0 (1.41), p = 0.156 and β (SE) = -2.0 (1.31), p = 0.127 respectively. Current smokers had higher depression scores than non-smokers with β (SE) = 2.0 (0.79), p = 0.011.Maternal history of depression was a strong predictor of PPD. Mothers previously prescribed antidepressants, β (SE) = 4.0 (0.99), p <0.0001 had worse depression scores.

Table 2.3 Univariate Median Regression Analysis for Postpartum Depression

Variable	n	β (SE)	p-value
Maternal blood arsenic	578	1.17 (0.60)	0.064
Maternal blood manganese	580	0 (0.82)	1
Maternal blood lead	580	0 (0.57)	1
Maternal age at birth	581	-0.105 (0.058)	0.07
Maternal education	581	-	0.001
Maternal education above high school	183	-3.0 (1.05)	0.005
Maternal education at least to high school	248	-3.0 (0.80)	0.0002
Maternal education, less than high school	150	-	-
Household income	387	-	0.039
Household income > $40,000	71	-2.0 (1.31)	0.127
Household income $20,001 - $40,000	129	-3.0 (1.04)	0.004
Household income $10,001 - $20,000	96	-2.0 (1.41)	0.156
Household income ≤ $10,000	91	-	-
Current smoker	582	2.00 (0.79)	0.011
Ever prescribed depression medication	575	4.00 (0.99)	<0.0001
Living with at partner	565	0.00 (0.90)	1

Table 2.4 shows the results of multivariable regression at the median. We found that maternal blood arsenic concentration at childbirth was significantly associated with depression with β (SE) = 0.967 (0.438), p = 0.027 after adjusting for maternal age, smoking, maternal education, household income, living with a partner, and having ever been prescribed antidepressant medication. Neither maternal blood manganese nor maternal blood lead were associated with depression at the 50^{th} quantile with β (SE) = -0.862 (0.744), p = 0.247 and β (SE) = -0.002 (0.587), p = 0.997 respectively. Maternal history of antidepressant use remained significant in the full model with β (SE) = 3.84 (0.97), p < 0.0001.

Table 2.4 Multivariable Median Regression Analysis for Postpartum Depression

Variable	β (SE)	p-value
Maternal blood arsenic	0.967 (0.438)	0.027
Maternal blood manganese	-0.862 (0.744)	0.247
Maternal blood lead	-0.002 (0.587)	0.997
Maternal history of antidepressant use	3.84 (0.97)	<0.0001

Model adjusted for age, maternal education, household income, living with a partner, smoking status and having ever been prescribed antidepressants.

As previously stated, the EPDS scores were abnormally distributed. To explore if effects of metals concentration varied depending on the quantile of depression scores, we also modeled our data at various quantiles of EPDS scores from the 25^{th} quantile to the 90^{th} quantile. Figures 2.1 to 2.3 show that the effect estimates of association between PDD and the arsenic, manganese and lead at different quantiles of depression scores.

Arsenic was positively associated with depressive symptoms. Shown in Figure 2.1, the association between maternal blood arsenic concentrations and PPD were statistically significant at the 25^{th}, 30^{th}, 40^{th}, 50^{th} and 60^{th}, 75^{th} quantiles with β (SE) = 0.586 (0.214), p = 0.006; β (SE) = 0.782 (0.249), p = 0.002; β (SE) = 1.07 (0.32), p = 0.001; β (SE) = 0.967 (0.438), p = 0.006; β (SE) = 0.971 (0.503), p = 0.054 and β (SE) = 1.03 (0.519), p = 0.047 respectively. Arsenic was marginally associated with depression scores at the 70^{th} quantile with, and β (SE) = 0.944 (0.498), p = 0.058. We did not find an association between arsenic and depression scores at the 80^{th} and 90^{th} quantiles of depression with β (SE) = 0.851 (0.519), p = 0.101 and β (SE) = -0.040 (0.684), p = 0.954 respectively.

Figure 2.1 The Association between Postpartum Depression and Arsenic Varies by Quantile

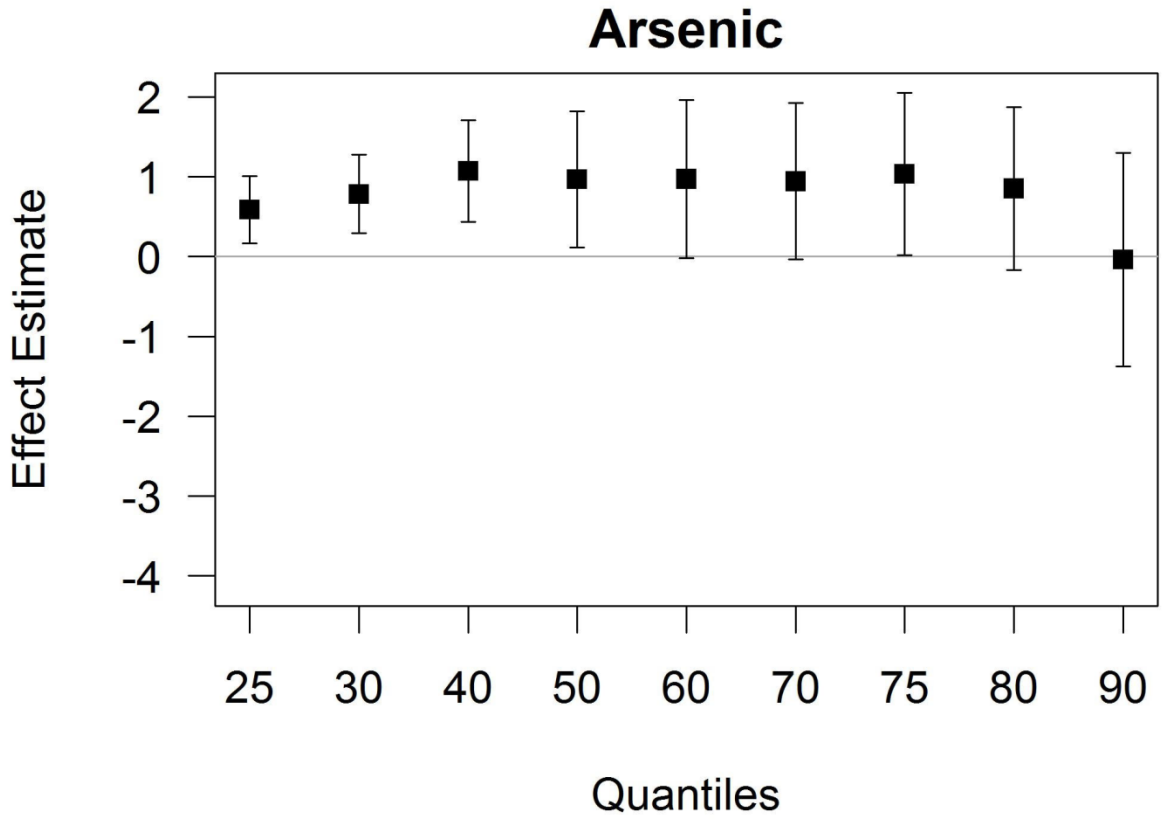
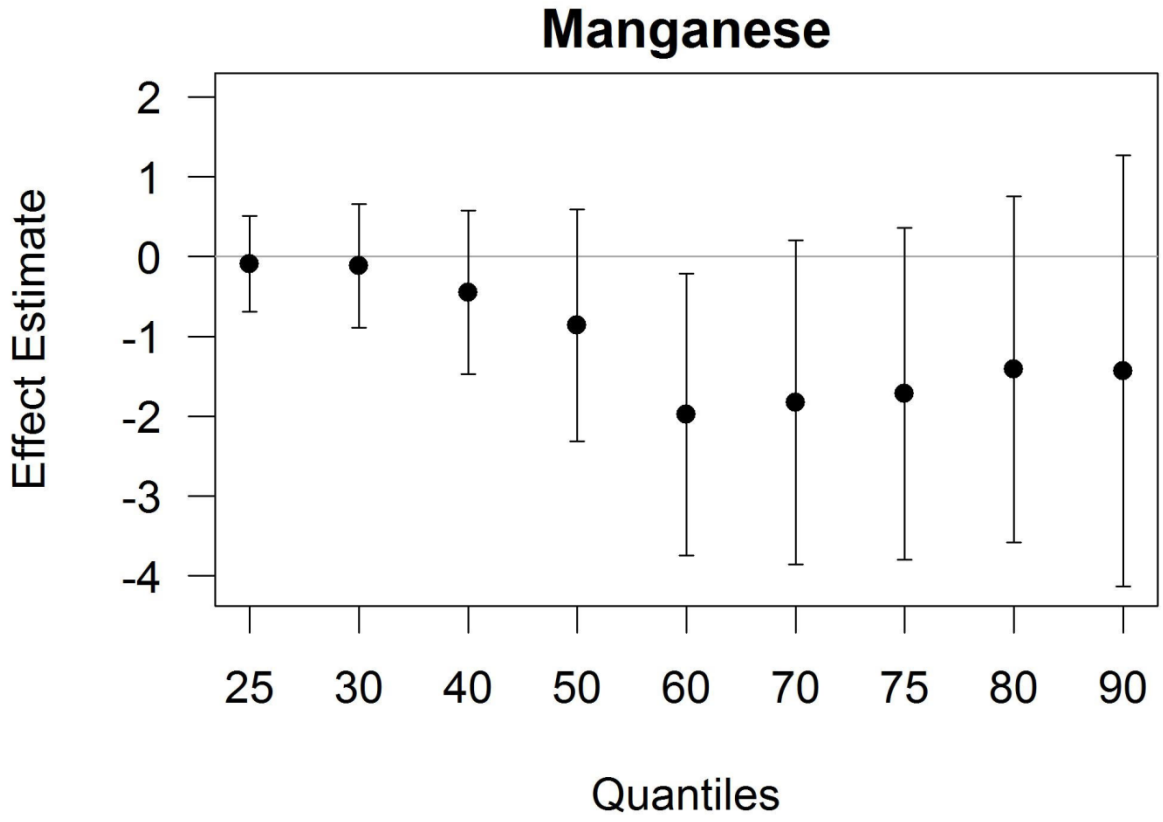


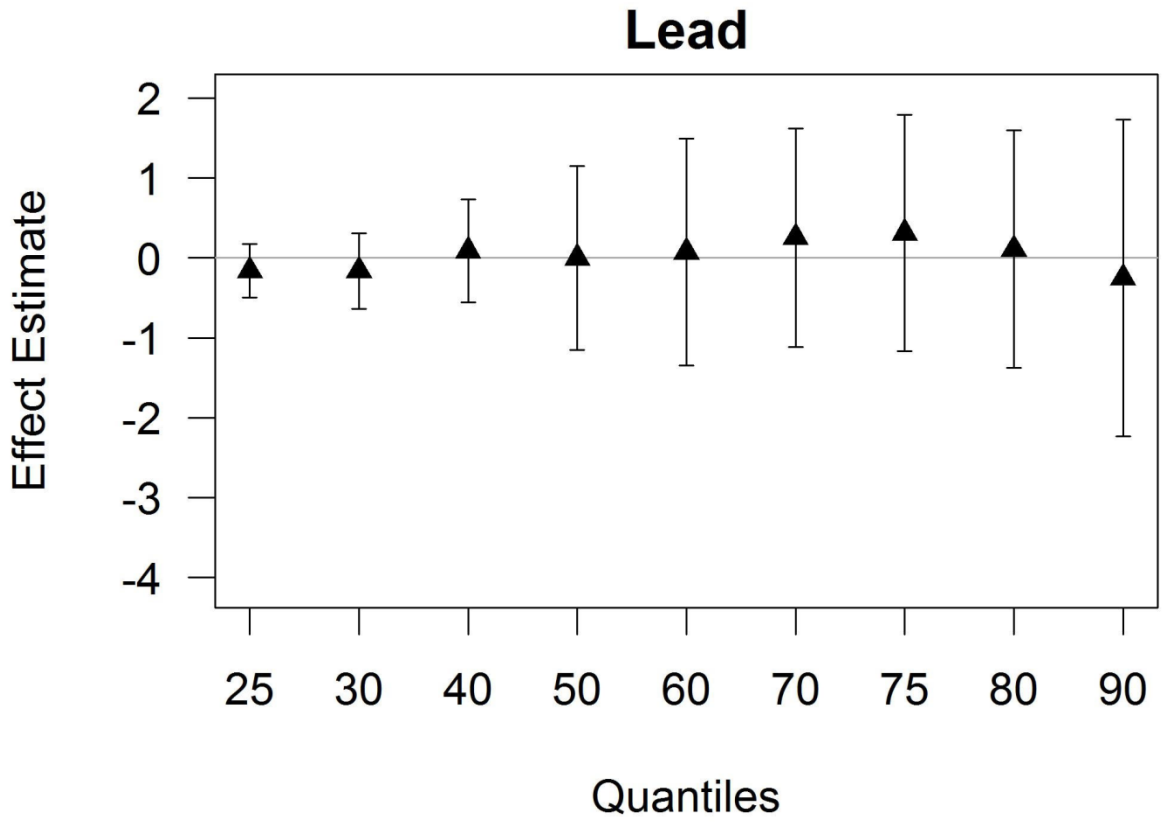
Figure 2.2 shows the estimates of the association between manganese and PPD. While the association between manganese and PDD scores was statistically significant at the 60^{th} quantile with β (SE) = -1.98 (0.90), p = 0.029. The association between manganese and PDD scores was marginally significant at the 70^{th} quantile with β (SE) = -1.83 (1.02), p = 0.074. We found no other significant associations at other quantiles of depression score. At the 25^{th} quantile, the association between manganese and EPDS scores was β (SE) = -0.093 (0.305), p = 0.762. The association at the 30^{th} percentile was β (SE) = -0.115 (0.397), p = 0.773. The estimate at the 40^{th} quantile was β (SE) = -0.449 (0.524), p = 0.392. The estimate at the 50^{th} quantile was β (SE) = -0.862 (0.744), p = 0.247. The associations at the 75^{th}, 80^{th} and 90^{th} quantiles were β (SE) = -1.72 (1.06), p = 0.105, β (SE) = -1.41 (1.11), p = 0.203 and β (SE) = -1.43 (1.36), p = 0.294 respectively.

Figure 2.2: The Association between Postpartum Depression and Manganese Varies by Quantile



As shown in Figure 2.3, we found no significant associations between blood lead levels and PPD scores in our study. At the 25th quantile, the association between manganese and EPDS scores was β (SE) = -0.163 (0.170), p = 0.339. The association at the 30th percentile was β (SE) = -0.163 (0.241), p = 0.499. The estimate at the 40th quantile was β (SE) = 0.088 (0.330), p = 0.790. The estimate at the 50th quantile was β (SE) = -0.002 (0.587), p = 0.997. The associations at the 60th, 70th, 75th, 80th and 90th quantiles were β (SE) = 0.072 (0.726), p = 0.921; β (SE) = 0.253 (0.698), p = 0.716; β (SE) = 0.311 (0.754), p = 0.681; β (SE) = 0.108 (0.759), p = 0.886 and β (SE) = -0.252 (1.009), p = 0.803 respectively.

Figure 2.3 The Association between Postpartum Depression and Lead by Quantile



In addition to the main effects, we investigated interactions between metals. As shown in Table 2.5, we found a significant lead-arsenic interaction at the 50th quantile (median) with β (SE) = 1.49 (0.62), p = 0.017. The main effect of arsenic was still significant with β (SE) = 0.80 (0.34), p = 0.018. The main effects of lead and manganese remained insignificant with β (SE) = -0.31 (0.45), p = 0.489 and β (SE) = -1.04 (0.81), p = 0.200 respectively. Again, the association between maternal history of antidepressant use and EPDS scores remained significant with β (SE) = 4.09 (0.85), p < 0.0001. Additionally, we found that mothers whose reported household income was ≤ $10,000 per year had higher depression scores than mothers who reported annual household incomes greater than $40,000 with β (SE) = 3.07 (1.19), p = 0.011.

Table 2.5 Interaction between arsenic and lead in PPD

Variable	β (SE)	p-value
Maternal blood arsenic	0.80 (0.34)	0.018
Maternal blood manganese	-1.04 (0.81)	0.200
Maternal blood lead	-0.31 (0.45)	0.489
Maternal blood arsenic*lead interaction	1.49 (0.62)	0.017
Household income ≤ $10,000/year	3.07 (1.19)	0.011
Maternal history of antidepressant use	4.09 (0.85)	<0.0001

Model adjusted for age, maternal education, household income, living with a partner, smoking status and having ever been prescribed antidepressants.

DISCUSSION

We found several interesting results in our study of the association between metals toxicity and postpartum depression. We found a significant positive association between blood arsenic concentrations and depression in mothers in our adjusted model. EPDS scores increased by 1.03 (0.45) points for every 1 unit increase in blood arsenic level above the mean (p=0.023). In addition, we found a significant interaction between arsenic and lead in our model $\beta = 1.49$ (0.62), $p = 0.017$. These results highlight the importance of looking at metal co-exposures when investigating their neurotoxicity.

At lower EPDS score quantiles of ($\leq 60^{th}$ percentile), manganese shows a protect effect on PPD. This protective effect lessens at higher quantiles of depression scores. One possible explanation is that mothers in higher quantiles of EPDS scores have competing risks for PPD. While we adjusted for some risk factors such as maternal history of smoking, age, marital status and maternal education, other risks factors exist.

Recent studies have suggested that depression may be the result of chronic systemic inflammation and oxidative stress (Rawdin et al, 2012 and Schiepers et al 2005). Studies have found that pro-inflammatory cytokines are associated with increased depression (Reus et al, 2015). A 2013 study by Vargas et al found that subjects with a history of suicide attempts had higher levels of nitric oxides ($p = 0.001$) and lower plasma total antioxidant potential ($p =0.005$) than subjects without a history of suicide attempts. These results further support studies that suggest that oxidative stress is a key mechanism in the neurotoxicity of arsenic, lead and manganese.

The results of our study are consistent with suggestions that arsenic is neurotoxic in adults even at low levels of exposure. Women of childbearing potential are particularly

susceptible to the adverse effects of environmental toxicants. These exposures are not only harmful to fetal development, but can have a significant impact on maternal mental health. This further compounds the detrimental effects of child development. More research is in the area of heavy metals and maternal mental health is needed.

Strengths

The current cross-sectional analysis focuses on the associations between low-level exposures to arsenic, lead and manganese and postpartum depression. Many previous studies, particularly those investigating arsenic toxicity are within populations with much higher arsenic exposure than seen in the US. Because our subjects have relatively low arsenic concentrations we avoid the extreme outward indications seen with arsenicosis. Participants are therefore unlikely to have suffered the same social isolation and stigmatization due to chronic arsenic toxicity seen in many international studies.

Many previous studies have relied on environmental measures of exposure such as analysis of drinking water samples. The use of biomarkers of exposure allows us to better estimate actual body burden on the individual level.

Limitations

In our current analysis, we do not make use of the longitudinal aspect of the study design. Instead, we focus on investigating the association of maternal metals concentrations and postpartum depression at delivery. By performing a cross-sectional analysis, we avoid the issues of loss to follow-up. On the other hand, we are not able to investigate the timing of exposure and it relationship to the development of depression symptoms. Missing data at follow-up could be due to population and area instability. Subjects submitted baseline EPDS questionnaires within hours of childbirth. Though hormonal changes occur within new moms within 48 hours of

delivery, our results may reflect antepartum depression as well as postpartum depression. We partially control for this by including previous history of depression (having ever been prescribed antidepressants) in our adjusted models. Still, there may be some residual confounding.

Using blood levels as a biomarker has its disadvantages. The half-life of arsenic and manganese in blood are in the order of hours. Use of blood is therefore not ideal in investigating chronic metals exposure. Analysis of other biomarkers such as hair may be best for future analyses.

We did not adjust for race in our analysis. Study participants are predominantly white and Native American population thus limiting the generalizability of the results to other ethnic populations. Finally, the final regression model may not capture residual confounding from other variables.

Future study

Future analysis will examine the association between metal and depression in mothers at 6 and 12 months postpartum. Doing so while allow us to explore the association between the progression of PDD and heavy metals. We will also analyze a sub-set of data using hair as a biomarker of exposure. Though analysis will involve a much smaller sample size, this may help to address concerns over the appropriateness of using blood as a biomarker. We will continue to analyze possible competing risk factors such as major life events that may shed light on the differences in the association between manganese and EPDS at different quantiles.

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CHAPTER IV

The Association between Arsenic and Plasma Total Histone 3 and H3K27me3 in a Neural Tube Defects Study

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ABSTRACT

Background: Arsenic exposures lead to various epigenetic changes. Previous studies have found that arsenic is associated with changes in histone levels. In addition, post-translational histone modifications (PTHM) have been identified in the brain tissue of children with neural tube defects (NTD).

Objective: Our objective was to investigate the association between arsenic exposure and histone levels in mothers of children with neural tube defects.

Design: We conducted a case-control study in Bangladesh. Cases were confirmed by physicians Controls were from the same area. We performed ELISA assays to investigate levels of total histone 3 and the histone modification H3K27me3 in maternal blood plasma.

Results: We found a significant association ($\beta = 0.041$, $p = 0.006$) between H3K27me3 levels and NTD case status. Among mothers with low folate, H3 was negatively associated ($\beta = -10.5$, $p = 0.016$) with maternal arsenic exposure.

Conclusion: Our results suggest that histone levels may differ among mothers of children with a NTD and those without NTD.

INTRODUCTION

Arsenic is a metal found globally in the earth's crust at varying concentrations. Exposure to arsenic primarily stems from contamination of drinking water (Tchounwou, 1999), direct exposure to soil, and inhalation of house dust (Zota, 2011). Current studies seek to elucidate the metabolic pathway of arsenic. Both animal and human studies suggest a couple of possible pathways. However, the exact mechanism of arsenic toxicity remains unknown.

Studies have linked arsenic exposure to numerous adverse health outcomes. Arsenic is classified as a 1 carcinogen (human carcinogen) according to the International Agency for

Research on Cancer (IARC). Numerous studies have investigated the neurological effects of arsenic. Though predominantly thought of as toxic to the peripheral nervous system, emerging evidence suggest that arsenic is also toxic to the central nervous system.

The neural tube, which eventually forms the spinal cord and brain, begins to develop around 21 days of gestation. The process involves fusion of tissue at various sites and the progressive closure from those sites to formation of an intact central nervous system. Neural Tube Defects (NTDs) affect one per 1,000 pregnancies worldwide (Greene et al, 2011).

Epigenetic mechanisms operate throughout the life cycle including fertilization and are critical in embryonic development. The epigenetic mechanisms of DNA methylation, micro RNA and post-translational histone modifications (PTHM) are important in gene regulation. During fetal development, DNA and histone methylation signals undergo programming and reprogramming (Pozharny, 2010).

Previous studies have shown that arsenic exposure affects epigenetic mechanisms. Many studies have investigated mechanisms involving DNA methylation. An *in vitro* study by Reichard et al, 2007 found that arsenic exposure repressed DNMT1 and DNMT3A expression and depleted the methyl donor, SAM. DNMT1 and DNMT3A are major DNA methyltransferases that affect gene expression via methylation (Feng et al, 2010). A 2012 study by Pilsner et al found that increasing maternal urinary arsenic was associated with increased global DNA methylation in cord blood.

Post-translational histone modifications are an important epigenetic mechanism. These modifications have an important role in chromatin structure and gene transcription. Some modifications work to activate transcription while others suppress transcription. PTM can include methylation, acetylation and phosphorylation (Schneider et al, 2007).

Recent studies have investigated post-translational histone modification (PTHM) as another epigenetic mechanism. A study by Zhou, Q et al, 2008 found that arsenite affected several histone modifications including increased H3K9 dimethylation and decreased H3K27 trimethylation in A459 cancer cells. In a Bangladesh study by Chervona et al,, 2012, researchers found that levels of H3K27me3 increased with increasing water arsenic among female adults but showed a negative association among males. The same study found total urinary arsenic was negatively associated with H3K9ac. Though most studies examine intracellular histones, studies have shown that histone modifications can be detected in plasma (Deligezer, U. et al, 2010). Particulate matter (PM_1) was found to have a positive association with extracellular H3K4me3 and H3K9ac levels (Cantone et al, 2013)

A study examining human fetal brain tissue found decreased H3K79me2 expression in brains with neural tube defects. Additionally, the study found a novel site, H2bK5me1, which showed no expression in brains with NTDs compared to normal tissue (Zhang, 2013). A study by Tsurubuchi, T et al, 2013 sought to identify early biomarkers of NTDs (n=6). Researchers found that H3K4me2/3 and H3K27me2 levels were higher in the amniotic fluid stem cells (AFSC) of a woman carrying a fetus affected with myelmeningocele**.** Higher levels of H3K27me3 were found in women carrying both myelmeningocele and anencephaly-affected fetuses. The woman carrying the myelmeningocele fetus also had decreased levels of H3K9ac and H3K18ac.

Limited information exists on the association between arsenic and epigenetic mechanisms in the context of NTDs. Our current analysis investigates the possible association between histone levels and arsenic toxicity in neural tube defects in case-control pilot study in Bangladesh (Mazumdar et al, 2015).

METHODS

Study Population

The study population has been described previously (Mazumdar et al, 2015). Briefly, case-control pilot study was conducted in 2013 in Bangladesh. Subjects were recruited from communities served by Dhaka Community Hospital (DCH). Eligible cases were children under the age of 1 year with a neural tube defect, specifically myelmeningocele). Controls came from the same communities but did not share family drinking wells. Controls were selected randomly using immunization records and matched on gender and age within 2 months. 57 cases and 55 controls were identified. Investigators collected information such as nutrition (including plasma folate levels), education, medical history, family history and smoking from interviews and questionnaires.

Arsenic Exposure

Maternal toenail arsenic

Stainless steel scissors were used to clip toenail samples. Samples were stored in a paper envelope at room temperature until analysis at the Harvard School of Public Health (HSPH), Trace Metals Laboratory in Boston, Massachusetts Samples were digested with nitric acid as described in Chen, et al, 1999. Digested toenail samples were analyzed for arsenic using Inductively Couple Plasma Mass Spectrometry (ICP-MS) according to?.

Maternal blood plasma collection

10 ml samples of maternal whole blood were collected in EDTA tubes within 30 minutes of blood drawing. Samples were centrifuged at 2,000 rpm for 12 minutes. Plasma was collected in 5 mL cryovials and stored at -20^{o}c. Plasma samples were shipped to HSPH. Samples were aliquoted and kept at -80^{o}c until analysis.

Histone Modification Analysis

The concentrations of total histone H3 and H3K27me3 were measured using sandwich enzyme-linked immunoabsorbent assay (ELISA). 96-well microplates (Fisher Scientific, Pittsburg, PA) were coated with histone H3 antibody (Abcam, Cambridge, MA) and incubated overnight at 4^{o}c. Plates were blocked with 3% milk in PBST for 1.5 hours at room temperature with agitation. After coating incubation, plates were washed with PBST. Histone standards (total histone H3 and H3K27me3) were made by diluting desired amount of recombinant protein (Active Motif, Carlsbad, CA) in MQ water. Two Quality control (QC) plasma samples were prepared by separately pooling 50 maternal plasma samples. 5 µL of plasma was diluted in MQ water for each sample. 100 µL of standards, QCs and assay samples were added to each well. The plates were incubated at room temperature with agitation for 1.5 hours. Following incubation, samples were washed with PBST. Polyclonal antibody (Active Motif, Carlsbad, CA) was added to each well and incubated for 1 hour at room temperature with agitation. Plates were then washed with PBST. Secondary antibody goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA) was added to each well and incubated for 1 hour without agitation. Following incubation with secondary antibody, wells were washed with TBST. TMB (Fisher Scientific, Pittsburg, PA) was added to each well. Reaction was stopped when desired samples reached desired color (10-20 minutes). 2 M H2SO4 was added to stop the reaction. The optical density was read at 450 nm on the Infinite 200Pro spectrophotometer using V_3.22_12/10_Infinite firmware. Of the 112 subjects, 88 total histone H3 and 64 H3K27me3 samples have been analyzed.

Statistical Analysis:

Data was analyzed using SAS 9.3. Samples that failed quality control standards were excluded from the analysis. The current analytical dataset includes 85 samples (45 cases, 40 controls). Maternal arsenic measurements were log-transformed for analysis to approximate a normal distribution.

We used general linear regression to model the association between total histone H3 and H3K27me3 levels and variables. Maternal arsenic concentrations were log-transformed and modeled continuously. H3K27me3 concentrations were normalized by total histone H3 and were reported as proportions (ng/μL of H3K27me3 / ng/μL of total histone H3). Total histone H3 was modeled as measured (ng/μL). Both total H3 and H3K27me3 were modeled continuously.

Previous study of this population found a significant interaction between reported folate use and water arsenic concentrations (Mazumdar, 2015). This finding further underlined the need to include folate in our full analytical models. We modeled folate as a dichotomous variable, low folate. Mothers with low folate had plasma folate levels < 2 ng/mL based on WHO criteria. We also performed a stratified analysis to investigate whether the association between plasma histones and maternal arsenic differed between mothers with adequate folate and those with low folate.

We conducted a conditional logistic regression analysis to investigate the association between histone levels as a predictor and case status as dichotomous outcome. Again, we modeled maternal arsenic and histone levels as continuous variables and folate as a dichotomous variable. Other variables of interest include maternal age, paternal age, vitamin use and measures of SES such as receiving an ultrasound during pregnancy.

We also performed a secondary analysis in which we included a variable for the ELISA plate analyzed. This analysis was performed to explore possible batch (plate) effects.

RESULTS

Table 3.1 shows the characteristics of the study population. The mean (SD) maternal age at enrollment was 24.2 (5.03). Fathers were older with a mean (SD) age of 31.8 (5.93). The mean maternal age at childbirth was 23.4 (5.04). 33% of the mothers have low folate. Mean (SD) folate was 3.87 (3.95) ng/mL. The majority of mothers (88.2%) received some sort of prenatal care, indicated by receiving an ultrasound during pregnancy. Mothers also report taking folic acid supplements (51.8%) and vitamins (37.7%) during pregnancy. 47.1% of mothers reported use of medication. Only one mother (1.18%) reported being a smoker.

Table 3.1. Characteristics of Bangladesh Neural Tube Defect Pilot Study Population, n = 87

Characteristic	Mean (SD) or %
Mother's age	24.2 (5.03)
Mother's age at birth	23.4 (5.04)
Father's age	31.8 (5.93)
Folate (ng/ml)	3.87 (3.95)
Low folate	32.9%
Ultrasound during pregnancy	88.2%
Maternal vitamin use	37.7%
Maternal medication use	47.1%
Folic acid supplementation during pregnancy	51.8%
Maternal smoking	1.18%

The median (SD) maternal toenail arsenic is 0.69 (3.96) µg/g with a range of 0.12 to 27.7 µg/g. The mean total plasma H3 and plasma H3K27me3 concentrations are 160 (43.1) and 31.8 (11.8) ng/µL respectively (Table 3.2)

Table 3.2. Maternal Arsenic Exposure and Plasma Histones

Variable	Mean (SD)	25th	Median	75th	Min	Max
Exposure						
Maternal toenail arsenic (µg/g)	2.19 (3.96)	0.384	0.694	2.29	0.115	27.7
Outcome						
Total H3 (ng/µL)	160 (43.1)	128	158.0	179	108	455
H3K27me3 (ng/µL)	31.8 (11.8)	24.1	29.20	37.4	10.70	71.70

In our univariate analysis (Table 3.3a) of total plasma H3, maternal arsenic was not associated with total H3 levels with β (SE) = -6.05 (3.91), p = 0.126. We did not find an association with low folate status or case status with β (SE) = 1.33 (10.0), p = 0.895 and β (SE) = 2.97 (9.43), p = 0.777 respectively.

In our full, adjusted model, we found no association between maternal toenail arsenic and total plasma H3 with β (SE) = -6.97 (4.13), p = 0.096. Again, we did not find an association with low folate status or case status with β (SE) = 0.613 (9.97), p = 0.951 and β (SE) = 7.53 (9.82), p = 0.446 respectively.

In our univariate analysis (Table 3.3b) of modified H3K27me3, maternal arsenic was not associated with H3K27me3 levels with β (SE) = 0.002 (0.006), p = 0.764. We did not find an association with low folate status with β (SE) = -0.017 (0.009), p = 0.267. We found a significant association with case status with β (SE) = 0.038 (0.009), p = 0.007. Controls have higher plasma H3K27me3 concentrations than cases.

In our full, adjusted model, we found no association between maternal toenail arsenic and H3K27me3 levels with β (SE) = -0.003 (0.006), p = 0.50. Again, we did not find an association with low folate status with β (SE) = -0.017 (0.015), p = 0.234. The significant association between H3K27me3 levels and case status remained significant with β (SE) = 0.041 (0.014), p = 0.006.

Table 3.3a. Association between arsenic exposure and plasma total histone 3

	Total H3			
Variable	**Crude Model**		**Adjusted Model***	
	β (SE)	**p-value**	**β (SE)**	**p-value**
Maternal arsenic	-6.05 (3.91)	0.126	-6.97 (4.13)	0.096
Low folate	1.33 (10.0)	0.895	0.613 (9.97)	0.951
Controls	2.67 (9.43)	0.777	7.53 (9.82)	0.446

* full model adjusted for folate status, arsenic exposure and total H3 levels

Table 3.3b. Association between arsenic exposure and plasma H3K27me3

	H3K27me3			
Variable	**Crude Model**		**Adjusted Model***	
	β (SE)	**p-value**	**β (SE)**	**p-value**
Maternal arsenic	0.002 (0.006)	0.764	-0.003 (0.006)	0.570
Low folate	-0.017 (0.009)	0.267	-0.017 (0.015)	0.234
Controls	0.038 (0.014)	**0.007**	0.041 (0.014)	**0.006**

* full model adjusted for folate status, arsenic exposure and case status

We also performed a stratified analysis to investigate whether the association between plasma histones and maternal arsenic differed between mothers with adequate folate and those with low folate.

We did not find an association between maternal arsenic and total H3 levels among mothers with adequate folate concentrations with β (SE) = -5.11 (5.89), p = 0.390 (Table 3.4a). However, among mothers with low folate, we found maternal arsenic was significantly associated with total H3 levels with β (SE) = -10.5 (4.05), p = 0.016. Among mothers with low folate, maternal arsenic is negatively associated with H3 levels. No association was found between total H3 levels and case status for either adequate or low folate groups with β (SE) = 9.80 (13.8), p = 0.482 and β (SE) = 2.78 (9.85), p = 0.780 respectively.

We did not find an association (Table 3.4b) between H3K27me3 levels and maternal arsenic for either adequate or low folate groups with β (SE) = -0.005 (0.007), p = 0.517 and β (SE) = -0.001 (0.012), p = 0.912 respectively. We found a significant association between H3K27me3 levels and case status among mothers with adequate folate levels with β (SE) = 0.046 (0.016), p = 0.007 but not among mothers with low folate with β (SE) = 0.030 (0.029) p= 0.306. Among mothers with adequate folate levels, controls had higher H3K27me3 levels.

Table 3.4a. Association between maternal arsenic and total H3 stratified by low folate status

Total H3				
Variable	**Adequate Folate**		**Low folate**	
	β (SE)	**p-value**	**β (SE)**	**p-value**
Maternal arsenic	-5.11 (5.89)	0.390	-10.5 (4.05)	**0.016**
Controls	9.80 (13.8)	0.482	2.78 (9.85)	0.780

Table 3.4b. Association between maternal arsenic and total H3K27me3 stratified by low folate status

H3K27me3				
Variable	**Adequate folate**		**Low folate**	
	β (SE)	**p-value**	**β (SE)**	**p-value**
Maternal arsenic	-0.005 (0.007)	0.517	-0.001 (0.012)	0.912
Controls	0.046 (0.016)	**0.007**	0.030 (0.029)	0.306

We conducted a conditional logistic regression analysis to investigate the association between histone levels as predictor and case status as an outcome. Overall, we found no significant associations with total H3 levels (Table 5a). The univariate and adjusted OR was 1.00 (0.99, 1.02). The univariate OR (95% CI) and adjusted OR (95% CI) for the association with maternal arsenic was 1.46 (0.96, 2.22) and 1.47 (0.96, 2.24) respectively. The univariate OR (95% CI) and adjusted OR (95% CI) for the association with low folate status was 0.71 (0.23, 2.25) and 0.70 (0.21, 2.32) respectively.

However, we found that H3K27me3 levels were significantly associated with case status (Tables 3.5b). The univariate OR (95% CI) and adjusted OR (95% CI) for the association with H3K27me3 levels was 999.9 (19.7, >999.9) and 999.9 (33.0, >999.9) respectively. The univariate OR (95% CI) and adjusted OR (95% CI) for the association with maternal arsenic was 1.46 (0.96, 2.22) and 1.82 (1.001, 3.30) respectively. The univariate OR (95% CI) and adjusted OR (95% CI) for the association with low folate status was 0.71 (0.23, 2.25) and 1.46 (0.32, 6.71) respectively.

Table 3.5a. Conditional Logistic Regression for Case Status and Total H3 levels

Variable	Unadjusted OR (95% CI)	Adjusted OR (95% CI)*
Maternal arsenic	1.46 (0.96,2.22)	1.47 (0.96, 2.24)
Total H3	1.00 (0.99, 1.02)	1.00 (0.99, 1.02)
Low folate	0.71 (0.23,2.25)	0.70 (0.21, 2.32)

* Full model adjusted for folate status, arsenic exposure and total H3 levels

Table 3.5b. Conditional Logistic Regression for Total Case Status and H3K27me3 levels

Variable	Unadjusted OR (95% CI)	Adjusted OR (95% CI)*
Maternal arsenic	1.46 (0.96,2.22)	1.82 (1.001, 3.30)
H3K27me3	999.9 (19.7, >999.9)	999.9 (33.0, >999.9)
Low folate	0.71 (0.23,2.25)	1.46 (0.32, 6.71)

*full model adjusted for folate status, arsenic exposure and normalized H3K27me3 levels

In our secondary analyses, we explored possible batch (plate) effects. We repeated the previous analyses looking at (1) histone levels as a continuous outcome (2) stratification by folate status and (3) case status as an outcome, but also included plate as a variable in our model.

In our full, plate-adjusted model (Table 3.6a) of total plasma H3, maternal arsenic was not associated with total H3 levels with β (SE) = -0.897 (3.71), p = 0.810. We did not find an association with low folate status or case status with β (SE) = 5.23 (8.69), p = 0.549 and β (SE) = -10.9 (9.62), p = 0.262 respectively.

In our full, plate-adjusted model (Table 3.6b) of modified H3K27me3, maternal arsenic was not associated with H3K27me3 levels with β (SE) = -0.004 (0.006), p = 0.566. We did not find an association with low folate status with β (SE) = -0.021 (0.014), p = 0.144. We found a marginally significant association with case status with β (SE) = 0.028 (0.016), p = 0.075. Controls had higher plasma H3K27me3 concentrations than cases.

Table 3.6a. Association between arsenic exposure and plasma total histone 3 in plate-adjusted model

Total H3		
Variable	**Plate-Adjusted Model**	
	β (SE)	**p-value**
Maternal arsenic	-0.897 (3.71)	0.810
Low folate	5.23 (8.69)	0.549
Case status	-10.9 (9.62)	0.262

Table 3.6b. Association between arsenic exposure and H3K27me3 in plate-adjusted model

	H3K27me3	
Variable	**Plate-Adjusted Model**	
	β (SE)	**p-value**
Maternal arsenic	-0.004 (0.006)	0.566
Low folate	-0.021 (0.014)	0.144
Case status	0.028 (0.016)	0.075

Again, we performed a stratified analysis to investigate whether the association between plasma histones and maternal arsenic differed between mothers with adequate folate and those with low folate.

Unlike the non-plate adjusted analyses, we did not find an association between maternal arsenic and total H3 levels among mothers with adequate or low folate concentrations with β (SE) = 0.931 (5.42), p = 0.864 (Table 3.7a) and β (SE) = -3.16 (2.99), p = 0.302 respectively. We did find a significant association between case status and total H3 levels in mothers with low folate, β (SE) = -16.9 (7.58), p = 0.036, but not in mothers with adequate folate, β (SE) = -8.17 (14.1), p = 0.566

Consistent with our non-plate adjusted analyses, we did not find an association (Table 3.7b) between H3K27me3 levels and maternal arsenic for either adequate or low folate groups with β (SE) -0.004 (0.007), p = 0.565 and β (SE) = -0.0004 (0.013), p = 0.976 respectively. We found a marginally significant association between H3K27me3 levels and case status among mothers with adequate folate levels with β (SE) = 0.034 (0.019), p = 0.074 but not among mothers with low folate with β (SE) = 0.017 (0.033) p= 0.605.

Table 3.7a. Association between maternal arsenic and total H3 stratified by low folate status in our plate-adjusted analysis

Total H3 in plate-adjusted model				
Variable	**Adequate folate**		**Low folate**	
	β (SE)	**p-value**	**β (SE)**	**p-value**
Maternal arsenic	0.931 (5.42)	0.864	-3.16 (2.99)	0.302
Controls	-8.17 (14.1)	0.566	-16.9 (7.58)	**0.036**

Table 3.7b. Association between maternal arsenic and H3K27me3 stratified by low folate status

H3K27me3 in plate-adjusted model				
	Adequate folate		**Low folate**	
	β (SE)	**p-value**	**β (SE)**	**p-value**
Maternal arsenic	-0.004 (0.007)	0.565	-0.0004 (0.013)	0.976
Controls	0.034 (0.019)	0.074	0.017 (0.033)	0.605

Again, we conducted a conditional logistic regression analysis for case status. Overall, we again found no significant association with total H3 levels (Table 3.8a). The adjusted OR (95% CI) was 0.84 (0.64, 1.09). The adjusted OR (95% CI) for the association with maternal arsenic and low folate status was 1.90 (0.68, 5.34) and 0.54 (0.04, 6.73) respectively. Again, we found that H3K27me3 levels were significantly associated with case status (Tables 3.8b). The adjusted OR (95% CI) for the association with H3K27me3 levels was 999.9 (33.0, >999.9). The univariate OR (95% CI) and adjusted OR (95% CI) for the association with maternal arsenic and low folate status was 2.62 (0.71, 9.65) and 1.41 (0.10, 21.0) respectively.

Table 3.8a. Conditional Logistic Regression in our plate-adjusted analysis

Variable	Adjusted OR (95% CI) in plate-adjusted model
Maternal arsenic	1.90 (0.68, 5.34)
Total H3	0.84 (0.64, 1.09)
Low folate	0.54 (0.04, 6.73)

Table 3.8b. Conditional Logistic Regression in our plate-adjusted analysis

Variable	Adjusted OR (95% CI) in plate-adjusted model
Maternal arsenic	2.62 (0.71, 9.65)
H3K27me3	999.9 (33.0, >999.9)
Low folate	1.41 (0.10, 21.0)

DISCUSSION

Our current study investigates the association between plasma histone levels arsenic and mothers of children with myelmeningocele (cases). The results of our study suggest that the epigenetic profile of mothers of cases may differ from those whose children did not have a NTD. A strength of this pilot study is the ability to look at a specific modification (H3K27me3) that has been shown in previous literature to be associated, separately, with both arsenic and neural tube defects. The study of *plasma* histone levels may provide an opportunity for important insight into possible mechanisms as well as use as a biomarker of detection. Another strengthen, is the use maternal biomarkers of arsenic exposure (maternal toenail arsenic) and maternal, which provides a more accuracy representation of arsenic exposure.

Our study found a significant association between H3K27me3 and NTD case status when adjusting for maternal arsenic and folate status. Controls had higher levels of H3K27me3. The result of our conditional logistic regression also supports this finding. No association was found between total H3 levels and case status. The results of my study are interesting considering the findings of Tsurubuchi et al, 2013 which found that mothers with NTD fetuses had higher levels of H3K27me3 and Chervona et al, 2012 which found the direction of the association between wAs and H3K27me3 levels differed by gender. These results suggest that further analysis of modified extracellular (plasma) histone levels may provide insight into the mechanisms of arsenic toxicity in neural tube defects. This underlines the importance of studying appropriate modifications when investigating the role of posttranslational histone modifications and neural tube defects.

Our stratification analysis supports previous research that folate is important in arsenic toxicity as well as the pathology of neural tube defects. The levels of plasma total H3 were lower

with increasing arsenic only among mothers with low folate (< 2 ng/mL). This suggests that with adequate folate, the effects of arsenic on total H3 levels disappear. However, an association between case status and H3K27me3 levels was found only in mothers with adequate folate levels. These results suggest that even with the presence of adequate folate, plasma H3K27me3 levels are associated with NTD. Since the association disappears among mothers with low folate, this may suggest that the effect of folate on NTD is independent of plasma H3K27me3 levels.

In our results of our plate-adjusted analyses, while statistically weaker, the direction and magnitude of the association between H3K27me3 and case status remained the same with or without adjusting for plate effects. However, the associations between total H3 and maternal arsenic were not consistent. This brings into question the appropriateness of adjusting for plate effects. The cases and controls were not evenly distributed among ELISA plates. In addition, controls had higher levels of arsenic than cases. The seeming plate effect on total H3 levels is likely an artifact that cannot be teased apart with our current analysis. Adjustment for plate/ batch effects is more appropriate when measurements can be repeated in such a way that minimizes any pattern of sample analysis among plates. Combined with reduced power from the introduction of another variable, it is not completely surprising that the results of the analyses with total H3 levels were unstable. This further strengthens our view that H3K27me3 levels are a more robust biomarker of NTD status than overall plasma H3 levels.

Overall, the results of our study suggest that the relationship between histone modifications and arsenic exposure in mother's with NTD affected children is complex and like other epigenetic mechanisms, are in need of further study.

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CONCLUSION

In my work, I have shown that low-dose exposures to arsenic, lead and manganese can affect neurological outcomes in mothers and children. Not only are arsenic and lead separately detrimental, studying co-exposures to these metals is also important when looking at neurotoxicity. Typically, the association between arsenic and health has focused on cancer outcomes. My work shows that arsenic toxicity goes beyond that of its carcinogenic properties. While arsenic and lead have no nutritional benefit, the association between manganese and neurological outcomes remain unclear.

In Chapter 2, I investigated the association between metals exposure and neurobehavioral outcomes in children in the Tar Creek Cohort. We found a significant association between prenatal lead concentrations and the scores of Adaptive Skills domain of the BASC-2 (β = -5.99, p-value 0.025). Our results further support previous research that has shown that a positive cognitive home environment is important to neurobehavioral outcomes as measured by the BASC-2 and BRIEF assessments. We also showed there are gender differences in some of the neurobehavioral sub-domains.

My dissertation helps to address a much-needed topic – mental health, in a vulnerable population. In Chapter 3, we investigated the effect of low-level metals on postpartum depression. The results showed that mothers with increased arsenic exposure are more likely to suffer from depression. We also found a significant interaction between lead and arsenic. These results further reiterate the need to study metal co-exposures. We also found an interesting association between manganese, depending on the quantile of EPDS scores. Manganese appears to have a protective effect for mothers who were at or below the 60^{th} percentile for EPDS scores. Using the data from the Tar Creek cohort allowed us to observe these associations in a US

population with significantly lower heavy metal exposures than typically studied in international studies.

Chapter 4 of my dissertation is an epigenetic investigation of the association between arsenic and neural tube defects. The analysis of post-translational histone modifications in this pilot study found a significant association between epigenetic markers and arsenic. Our results also suggest that the epigenetic profile of mothers of children born with neural tube defects may differ from mothers whose children did not have a NTD. Much of the information we know about arsenic neurotoxicity relies on animal studies. My dissertation is a step showing that epigenetic mechanisms may help to explain arsenic toxicity in neurodevelopment.

Suggestions for Further Research

The longitudinal design of the Tar Creek cohort allows us to investigate the association between biomarkers of arsenic, lead and manganese exposure and other neurological outcomes.

Data from additional neuropsychological instruments including intelligence, memory and motor skills are available for children in the Tar Creek cohort. An examination of the association between other domains such as memory and motor skills and metals exposures may provide further insight. An important continuation for this study is to incorporate data from the children's neurological outcomes and postpartum depression. This may provide a richer understanding of the role of chemical toxicants and maternal mood in healthy child neurodevelopment.

Data from repeated measures of postpartum depression are available for this cohort. Future analyses will investigate the association between arsenic, lead and manganese levels and EPDS scores at 6 and 12 months. This may shed light onto the role of these metals in the

progression of PDD. Additionally, other instruments available delve more deeply into the psychosocial environment of the mothers and children; data is available on other factors including stress, anxiety and discrimination. This data may aid in our understanding of why beneficial effects of manganese up to the 60th percentile of EPDS cease for mothers at higher quantiles of EPDS scores.

Our Bangladeshi pilot study of the association between arsenic and histones is an important first step. While DNA methylation is an important epigenetic mechanism likely involved in arsenic toxicity, our study shows that another epigenetic mechanism, the role of histone modifications may also prove useful to our understanding. Future studies should focus on additional modifications related to arsenic toxicity such as H3K9ac.